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High temperature stress responses of Salvia splendens and Viola X wittrockiana

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HIGH TEMPERATURE STRESS RESPONSES OF *SALVIA SPLENDENS* AND *VIOLA X WITTROCKIANA*

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ABSTRACT

One of the greatest impediments affecting growth and development of bedding plants is high temperature stress. The deleterious effects of high temperature stress are the most pronounced during plug transplant and/or during the transition period from the nursery to the landscape. High temperature stress responses were investigated in *Salvia splendens* and *Viola x wittrockiana* to determine the morphological and physiological mechanism associated with the heat tolerance. Two genotypes for each species were used; a heat tolerant Vista and a heat sensitive Sizzler cultivar of salvia and Crystal Bowl Purple (CBP) a heat tolerant and Majestic Giant Red (MGR) a heat sensitive cultivar of pansy. Morphological traits such as short stature, greater total leaf area/plant, extensive root system; physiological traits like stomatal conductance, greater transpiration, and net photosynthesis were found to be characteristic traits of heat tolerant cultivars. Greater cell membrane thermostability (CMT) and expression of a heat shock protein of low molecular weight approximately 27kD may be responsible for heat tolerance in Vista and CBP. Primary soluble sugars sucrose and raffinose found in both the salvia cultivars, and greater concentrations in Vista at high temperatures, may be involved in stabilization of membrane lipid bilayer and/or acting as osmoprotectants under stress conditions.

Short duration (3 h) heat preconditioning at 35 °C and 30 °C for salvia and pansy respectively during initial stages of growth helped to develop acquired thermotolerance. Heat sensitive cultivars acquired heat tolerance with heat preconditioning and acclimated to subsequent challenging temperatures. Preconditioning enhanced already existing traits of heat tolerant cultivars. Increased stomatal frequency and leaf thickness in salvia acquired during preconditioning.

Cell membrane thermostability measured at a single temperature with increasing time of exposure was closely associated with increased gas exchange rates, leaf relative water content and overall marketable quality in salvia. These simple laboratory techniques to test heat tolerance have a greater advantage over time and space consuming field trials and may be an accurate and more efficient measure of heat tolerance.

CHAPTER 1. INTRODUCTION

Herbaceous annuals and perennials have been the fastest growing sector of ornamental production in the United States (Nordwig and Erwin, 1996). Bedding plants include a large number of annual and perennial plant species that provide seasonal color to landscapes and home gardens. Bedding plants provide a wide variety of flower colors and textures for landscaping and they are the primary source of income for the floriculture industry. The largest consumers of bedding plants are the general public and the second largest are landscape companies (Copes, 2000). Among this diverse variety of bedding plants; the annuals salvia (*Salvia splendens*) and pansy/viola (*Viola x wittrockiana*), and the perennials coreopsis (*Coreopsis grandiflora*) and gaillardia (*Gaillardia x grandiflora*) are considered some of the most commonly observed plants in the landscape. Because of the continued increase in demand for these types of plants, the industry has been seeking more information/research on production and viability of these plants.

According to the USDA Floriculture Crops Summary for 2004, the total floriculture crop value at wholesale for all growers with $10,000 or more in sales was estimated at $5.18 billion. The total wholesale value of floriculture crops grown by operations exceeding the $100,000 sales level was $4.89 billion. Bedding and garden plants wholesale value of $2.53 billion was the largest contributor to the value of floriculture production. Among the specific bedding plants in the survey, pansy/viola flats contribute the second largest amount of $109 million next to impatiens.

The amount of breeding work conducted with bedding plants continues to increase as exemplified by the development of a large number of new or improved series

and varieties every year. Many bedding plants, however, are not suitable for production throughout the year. In transition regions like mid-south and southern Gulf States, cool-season plants are especially difficult to manage during late summer months and early fall (Wehner and Watschke, 1987). This lack of adaptation to changing climates often results in reduced marketable quality and failure to survive in landscapes.

One of the greatest impediments affecting growth and development of these plants is high-temperature stress. Heat stress is associated with conditions where temperatures are hot enough for a sufficient time that they cause *irreversible* damage to plant function or development (Hall, 2001). The deleterious effects of high temperature stress are most pronounced during plug transplant and/or during the transition period from the greenhouse to the landscape. The capacity of plants to acclimate and maintain normal physiological activities under high temperature is a critical factor in heat tolerance (Hale and Orcutt, 1997). This adaptation/acclimatization to high temperature is considered as one of the most important determinants for geographical distribution of plants (Mahan et al., 1997). The effects of heat stress and the ability of plants to resist stress can be an important factor in plant growth and survival in stressful environments (Lichtenthaler, 1996).

A long-term solution to the heat stress problem in ornamental bedding plants will require understanding the morphological and physiological responses. To date, the heat tolerance of bedding plants has been evaluated only under landscape situation by regional trials (Albrecht and Pair, 1994; Bailey, 1998; Pemberton and Robertson, 2001). Physiological data are lacking regarding whole plant heat tolerance of ornamental plants. Therefore, the primary objectives of this research were to investigate the effects of heat stress on the growth and physiological responses of herbaceous annuals and perennials.

The specific objectives were as follows:

1) Study short duration high temperature exposure on leaf gas exchange, cell membrane stability and heat shock protein expressions in

2) Screening of four bedding plant cultivars of salvia (*Salvia splendens*) 'Vista Red' and 'Sizzler Red'; pansy (*Viola x wittrockiana*)'F1 Iona', 'F1 Nature'; coreopsis (*Coreopsis grandiflora*) 'Sunray' and gaillardia (*Gaillardia x grandi flora*) 'Goblin' for heat tolerance by cell membrane thermostability test (CMT) and heat shock protein expression.

3) Study the short duration high temperature stress effects on plant growth, leaf gas exchange rates, total soluble sugars, small heat shock proteins and CMT test for screening for heat tolerance in heat tolerant and heat sensitive bedding plant cultivars.

4) Study the effect of short duration heat preconditioning on acquired morphological, leaf anatomical and physiological heat tolerance in heat tolerant and heat sensitive cultivars of salvia and pansy.

5) Investigate and test a technique for screening bedding plants for heat tolerance under laboratory condition and correlate with marketable quality of plants.

CHAPTER 2. LITERATURE REVIEW

Supraoptimal temperatures can reduce plant growth, development, and yield (Gusta et al., 1997; Harding et al., 1989). This adverse effect of high temperature can be deleterious to establishment of young seedlings that have been transplanted for commercial flowering plants or in landscapes. Blake et al. (1983) reported that newly transplanted confiner seedlings resulted in slow growth and increased mortality when transpirational water loss exceeded the capacity of roots to absorb moisture. During periods of high air temperatures, soil temperatures often reach injuriously high levels affecting root growth, strongly influencing shoot growth, leaf senescence and survival of whole plants (Aldous and Kaufmann, 1979; Kuroyanagi and Paulsen, 1988; Paulsen, 1994; Udomprasert et al., 1995, Xu and Huang, 2004). Several studies indicated that leaf injury under supraoptimal temperatures was due to direct inhibition of root growth, hormonal perturbation and its transport, water and nutrient uptake (Graves et al., 1991; Huang et al., 1991; Kramer, 1983; Gur and Shulman, 1979; Huang and Xu, 2000; Huang, 2001, Xu and Huang, 2004).

Like almost all other growth processes, photosynthesis is strongly affected by temperature. In most plants, changes in photosynthetic rates with response to temperature are reversible over a considerable range (10 °C to 35 °C), but exposure to temperatures below or above this range may cause irreversible injury to the photosynthetic system (Berry and Bjorkman, 1980). The common symptoms observed due to high temperature injury are appearance of necrotic lesions, chlorotic mottling of leaves, flowers, fruits and death (Larcher, 1995).

Inhibition of photosynthetic CO_2 fixation under high temperature conditions has been documented in many plant species including salvia (*Salvia splendens*). Under

ambient conditions of CO_2 and O_2, photosynthesis and the export rates of assimilates in salvia declined as leaf temperature was raised from 25 to 40 °C (Jiao and Grodzinski, 1996). Several components of the photosynthetic apparatus and associated metabolic processes are heat labile. Photosystem II (PSII), the most sensitive of the chloroplast thylakoid-membrane protein complexes involved in photosynthetic electron transfer and ATP synthesis, is the most thermolabile photosynthetic process (Berry and Bjorkman, 1980; Havaux, 1993). Leaf temperature above 35 °C for example resulted in thermal uncoupling of chloroplast thylakoids, inactivation of photosystems and inhibition of phosphorylation in cucumber (*Cucumis sativus*) and rice (*Oryza sativa*) seedlings (Taub et al., 2000; Vani et al., 2001). Bauer and Senger (1979) reported a non-stomatal inhibition of CO_2 uptake after heat stress by a strong increase of the CO_2 compensation concentration in ivy leaves (*Hedera helix* L.). In an experiment conducted by Ranney and Peet (1994) heat stress in five taxa of Birch (*Betula* species) resulted in a differential responses of source activity and sink capacity of the plant as temperature treatment increased from 25 to 40 °C. River birch (*B. nigra* L.cv Heritage) maintained highest net photosynthesis at the high temperature, while lowest was in paper birch (*B. papyrifera*).

Another study reported that reduced partitioning of photoassimilates in tubers resulted in reduced yield of potato due to high temperatures (Ewing, 1981). Thus export of photoassimilates is another metabolic process that is sensitive to inhibition by high temperature. Jiao and Grodzinski (1996) reported that heat stress inhibited assimilate export to a greater degree than net photosynthesis in salvia (*Salvia splendens*).

The ability of plants to withstand high temperature stress and continue to produce optimal growth is due not only to maintaining a superior photosynthetic performance at high temperatures, but also maintenance of membrane integrity. A cellular membrane

system is considered to be central to heat tolerance in plants (Raison et al., 1980). Cell membrane thermostability can be determined by measuring the electrical conductivity of water surrounding leaf tissue subjected to heat stress. The method is rapid and inexpensive for determination of heat tolerance of several genotypes. Cell membrane stability has been correlated with whole plant heat tolerance in several crops including soyabean (*Glycine max*) (Martineau, 1979), sorghum (*Sorghum vulgare*) (Sullivan and Ross, 1979), wheat (*Triticum aestivum*) (Saadalla et al., 1990), potato (*Solanum tuberosum*) and tomato (*Lycopersicum esculentum*) (Chen et al., 1982) and turfgrass (Marcum, 1998).

Heat tolerance of plants in larger part could be a result of an increased heat stability of the photosynthetic apparatus and thermotolerance of PSII. Both of these are dependent on the thylakoid membrane stability that varies widely among species and also varies according to acclimation of PSII to heat stress (Berry and Bjorkman, 1980; Weis and Berry, 1988). Positive associations between membrane stability and grain yield under heat stress have been reported for two spring wheat (*Triticum aestivum* L) populations (Blum et al., 2001). There has been some work on membrane stability and enhancing heat tolerance of other crops; for example recombinant inbred lines of wheat (*Triticum aestivum*) showed a positive correlation between membrane stability and grain yield of plants grown under hot summer conditions (Blum et al., 2001). Fokar et al. (1998) reported correlation of membrane stability with seedling heat tolerance in four hot environments in Mexico, Sudan, India and Brazil in wheat (*Triticum aestivum*). For cowpea, electrolyte leakage or membrane damage of leaf discs was negatively correlated with reproductive-stage heat tolerance (Ismail et al., 1999). Subsequent genetic selection

experiments by Thiaw and Hall (2004) confirmed that leaf electrolyte leakage under heat stress was negatively correlated with heat tolerance for pod set in cowpea.

Another signature response of plants to high temperature stress is decreased production of normal proteins and an accelerated increase in production of a special group of proteins called heat shock proteins (HSPs). One of the most widely studied aspects of plant thermotolerance is enhanced expression of HSPs. Several physiological and biochemical processes such as stabilizing the membrane bilayer liquid crystalline state (Balogi, 2003) and chaperone function (Hassane et al., 2002) are triggered by synthesis and localization of these proteins (Cushman and Bohnert, 2000). Heat shock proteins comprise some of the most highly conserved protein families known. Organisms induce HSP synthesis when their temperature increases above that which is optimal for them, rather than at a universal temperature threshold (Parsel and Lindquist, 1993). The major role of HSPs involves stabilization of proteins in a particular state of folding, transport of proteins across membranes, assembly of oligomeric proteins, and modulation of receptor activities. Based upon these activities HSPs have been termed as "molecular chaperones" (Ellis, 1987).

Voluminous research indicates that HSP are involved in altering specific biochemical process necessary for adaptation (Neumann et al., 1989; Holmstrom et al., 1994; Hayashi, 1997; Deak et al., 1999). Several studies have been conducted to assess the relative synthesis of HSPs in heat tolerant and heat non-tolerant plants. For example two different lines of creeping bent grass (*Agrostis palustris* Huds) differing in heat tolerance subjected to 40 °C heat stress treatment significantly differed in HSP25 expression (Park et al., 1996). Little is known about protective adaptation of photosynthetic process to heat stress; however evidence suggests that chloroplast HSPs

are involved in photosynthetic and PSII thermotolrance (Heckathorn et al., 1998). Preczewski et al. (2000) and Downs et al. (1999) reported a positive correlation between ability of photosynthetic systems to withstand heat tolerance and amount of chloroplast HSP accumulation in tomato (*Lycopersicum esculentum*) and spinach (*Chenopodium album*). Increased levels of low molecular weight (lmw) HSPs are positively correlated with increased thermotolerance of PSII (Clarke and Critcheley, 1994). Also, greater production of chloroplast lmw HSPs, both within and among species, is positively correlated with whole-plant thermotolerance (Park et al., 1996). Colombo et al. (1992) reported that there was a relative difference in HSP synthesis in heat tolerant and heat sensitive clones of black spruce (*Picea mariana*), heat tolerant groups synthesized higher constitutive levels of HSPs compared to heat sensitive clones.

Genetic differences in high temperature sensitivity in sorghum (*Sorghum vulgare*) are correlated with variations in capacity for HSP synthesis (Ougham and Stoddart, 1986). Krishnan et al. (1989) reported a correlation between the synthesis of specific sHSP and the degree of thermotolerance expressed in wheat (*Triticum aesitivum*) following exposure to high temperatures. A difference in HSP synthesis and heat tolerance suggests that these traits are under genetic control. Small HSPs are the most abundant group of HSPs in plants, whereas in other organisms high molecular weight HSPs are the most abundant (Vierling 1991; Parsel and Lindquist, 1993). Plants typically produce more than 10 lmw HSPs in response to heat stress, with each HSP belonging to one of five distinct gene classes. Two of these classes encode cytosolic/nuclear proteins, one class encodes an endomembrane protein, one class encodes a mitochondrial protein, and another class encodes a protein that localizes to plastids (Waters, 1995). A study conducted in apple (*Pyrus pumila*) tissue treated at 26 to 40 °C for 36 h demonstrated in

vitro that both the chloroplast HSPs and the plant mitochondrial HSPs protect electron transport during heat stress in these organelles (Downs and Heckathorn, 1998). Evidence for induced thermal tolerance for transgenic tobacco seedlings (*Nicotiana tobaccum*) with over expression of plant cytosolic lmw HSPs was demonstrated by Park and Hong (2002). A strong correlation between level of thermal tolerance and sHSP expression in transgenic tobacco seedlings exposed to temperature of 40 and 45 °C for 4 h was observed.

There are experimental evidences to prove that in many tree species and agronomic plants when exposed to sub-lethal high temperatures (heat pre conditioning/heat acclimatization) have shown a reduced heat injury. Koppenaal et al. (1991) have shown that jack pine (*Pinus banksiana*), white spruce (*Picea glauca*) and black spruce (*Picea mariana*) seedlings exhibit reduced heat injury after heat preconditioning. Preconditioning of white spruce (*Picea glauca*) for 5 h at 38°C protected seedlings against heat damage under subsequent heat stress treatment. Damage was evaluated based on chlorophyll fluorescence, visible needle damage and ability to use NADPH and ATP in Calvin cycle with or without heat preconditioning and 30 min exposure to heat stress of 42-50 °C (Bigras, 2000). Prior exposure of plants to stressful environments resulted in increased tolerance to stress in various crop species. Seedling survival and recovery growth increased after 2 h induction treatment at 42 °C prior to different challenging temperatures in seedlings of sunflower (Senthil et al., 2003). Kentucky blue grass (*Poa pratensis*. L) after two cycles of drying and re-watering protected seedlings when subjected to continuous 35/30 °C heat stress treatement for 21 days (Jiang and Huang, 2001). Similar results were reported in cotton (*Gossypium species*) (Brown and Thomas 1980), perrenial rye grass (*Lolium perenne* L) and annual

blue grass (*Poa annua*) (Wehner and Watchke, 1987). High temperature induced heat tolerance has not been studied extensively as drought induced heat tolerance. Moreover, there has been no work done with stress induced heat tolerance in ornamental bedding plants. Most of the studies conducted were concentrated on turf grass species Kentucky blue grass (*Poa pratensis* L), blue grass (*Poa annua*), perennial rye grass (*Lolium perenne* L), and creeping bent grass (*Agrostis palustris* Huds) etc, and other commercial crops such as soyabean (*Glycine max*) and cotton (*Gossypium hirsutum*). Understanding the relationship between morphological, physiological and anatomical responses of heat stress on preconditioning and subsequent heat stress acclimation would help to provide management practices for bedding plant production.

The studies listed in this review indicate that high temperature stress affects various morphological, physiological and molecular responses within plants. Effect of high temperature stress not only affected vegetative and reproductive growth, but also deleteriously affected root growth and storage of photoassimilates. High temperature can affect the marketability of herbaceous perennial and annual ornamental plants through adverse effects on source-sink relationships and cause damage during nursery stock production, stock handling and after planting. Understanding the morphological, physiological and anatomical ability of plants to tolerate high temperature stress may be important for recommending production protocol and for plant improvement programs.

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CHAPTER 3. SCREENING FOR HEAT TOLERANCE IN BEDDING PLANTS

INTRODUCTION

Herbaceous annuals and perennials are the fastest growing ornamental sectors in the United States (Nordwig and Erwin, 1996). One of the greatest impediments affecting growth and development of these plants is high-temperature stress. Heat stress is associated with conditions where temperatures are hot enough for a sufficient time that they cause *irreversible* damage to the plant function or development (Hall, 2001).

Nursery producers and greenhouse growers of bedding plants have little research based recommendations to guide them to alleviate various effects of environmental factors on growth and to determine best production practices for quality plant production (Hancheck and Cameron, 1995). Heat stress is one of the primary environmental factors that affect several physiological plant processes such as photosynthesis, respiration, protein function, translocation, and membrane permeability (Bauer, 1978; Chaisompongpan, et al., 1990). Plants differ in their ability to survive high temperatures, and thermotolerance depends upon their genotype and environmental requirements for optimum growth (Kerting, 1984; Gusta et al., 1997).

Cell membrane modification is one of the primary changes that occurs during plant stress leading to disfunction (Raison et al., 1980). Cellular membrane dysfunction caused by stress results in significantly increased permeability of ions and electrolytes, which can be readily measured by the efflux of electrolytes (Raymond et al., 1986; Hallam and Tibbits, 1988). Hence membrane dysfunction can be measured by determining electrical conductivity (EC) of cellular leakage from heat stressed leaf tissue. This is a rapid laboratory technique to assess plant heat tolerance (Wu and Wallner, 1993;

Yeh and Hsu, 2004). This technique is commonly termed cell membrane thermostability (CMT) tests.

Another major universal response of high temperature stress in almost all living organisms, including plants, is induction of heat shock proteins (HSPs) (Vierling 1991). HSPs exhibit highly ubiquitous and conserved features from bacteria to higher animals, and they are proposed to be essential for cell survival (Lindquist and Craig, 1988). In higher plants, the low molecular mass HSP class (15-42Kd) or small HSP (sHSP) are abundant and act as molecular chaperones (Basha et al., 2004). Low molecular mass HSPs have been identified in many diverse species, among both monocots and dicots (Kloppstech et al., 1985; Vierling et al., 1986, 1989).

The objectives of this experiment were to compare the thermotolerance of different cultivars of two herbaceous annuals (salvia and pansy/viola) and two herbaceous perennials (coreopsis and gaillardia) by 1) studying membrane damage of leaf tissues subjected to different temperatures using the CMT test and (2) compare the levels of heat shock protein synthesis in different plant parts exposed to sub lethal temperatures for different durations of times.

MATERIALS AND METHODS

Plant Material

The following plants were used in this study: *Salvia splendens* Vista series 'Red' and 'Purple' (heat tolerant) and Sizzler series 'Red' and 'Purple' (heat sensitive); pansy *Viola x wittrockiana* F1 Nature series 'Yellow' (heat tolerant) and F1 Iona series 'Yellow' (heat sensitive) and; *Coreopsis grandiflora* 'Sunray' (heat sensitive) and *Gailardia x grandiflora* 'Goblin' (heat tolerant). Seeds for this research were obtained from Pan American (West Chicago, IL) and American Takii (Salinas, CA). Seeds were

germinated in growth chambers (EGC, Chagrin Falls, OH) maintained at 24 /18 °C (day /night) and at a light intensity of 400 μ mol m^{-2} s^{-1} PPFD (photosynthetic photon flux density) for14-h photoperiod, and 75-80 % relative humidity. Plants in the growth chambers were fertigated every morning at approximately 0900H with 100 mg/L 15N-2.2P-12.4K (15-5-15 Cal Mg, Scotts-Sierra, Marysville, OH).

Heat Stress Treatments

The heat stress treatment was applied by gradual increments in temperature over a 2 h period starting at 24 °C and increasing at the rate of 4 °C every 30 min until reaching a maximum of 40 °C. The plants were held at 40 °C for a 3 h, 6 h or 12 h duration (Table 3.1). Relative humidity was maintained at 75-80 % during the heat stress treatments and all plants were well watered to prevent water stress. Leaves, stems and roots were harvested separately and immediately dipped in liquid nitrogen and stored at –80 °C until further analysis.

Table 3.1 Heat stress treatments applied for 3, 6 or 12 h at 40 °C.

Sub-lethal Heat Stress Treatments	
Time	**Ascending temperature**
0 min	24 °C
30 min	28 °C
60 min	32 °C
90 min	36 °C
120 min	40 °C
3 h, 6h or 12 h	40 °C

Membrane Damage

Electrical conductivity of membrane leakage was measured according to the method previously described (Raymond et al., 1986) with some modifications. Eight week old intact plants were exposed to moderate heat stress of 30 to 35 °C for at least 24 h before the test in order to allow for hardening (acclimation) in the programmed growth chamber. Five mm diameter discs of fully developed leaves were used for the electrolyte leakage test. Approximately 0.25g of leaf discs, were washed twice with distilled deionized water and incubated at 25 to 60 °C in glass tubes covered with saran wrap for 1 h in thermostat controlled water baths maintained at different temperatures. After tissue incubation the test tubes were cooled to room temperature and 25 ml of distilled deionized water was added to the flask for incubation at 25 °C for 1 h, under agitation. Water conductivity was measured using an electrical conductivity meter (VWR Scientific Instruments, Suwanee, GA). To calculate the maximum membrane damage, the test tubes containing the leaf discs were autoclaved for 15 min and the conductivity of the water was measured. These conductivity measurements were used to determine the lethal temperature (LT_{50}). LT_{50} is defined as the temperature at which there is 50% membrane damage determined by the conductivity of water surrounding the tissues treated at different temperatures. The percentage of membrane damage was calculated according to the following equation:

$$\text{Percent membrane damage} = \frac{Cx - Cc}{Cm - Cc} \times 100$$

Where C*x* is the conductivity of the water in which the samples are incubated at different temperatures for 2 h, C*c* is the conductivity of the water of the sample at 25 ^{o}C,

and C*m* is the maximum conductivity of the water when the samples are autoclaved for 15 min.

Protein Extraction

Frozen tissue samples from both heat stressed and non heat stressed plants were ground to fine powder using liquid N_2 and extracted using the protocol of Downs et al. (1998). Total protein from plant tissue was extracted in buffer containing Tris-HCL (pH 8.0), 1% sodium dodecyl sulfate (SDS: w/v), 1% dithiothreitol (DTT; w/v), 1mmol/L phenyl methyl sufonylfluoride (PMSF) and 5µmol/L Na_2EDTA. Polyvinylpyrrolidone (PVP) 3% w/v was used to remove phenolics. Samples were boiled for 3 minutes and centrifuged at 14000g for 6 min. The supernatant was collected and stored at –40 °C.

SDS-PAGE and Western Blot Analysis of Proteins

Aliquots of each sample containing 30µg of total protein (determined by BioRad Bradford assay) (Bradford,1976) were fractionated on 10% SDS-PAGE gels (Laemmli,1970) and electrophoretically transferred to a nitrocellulose membrane in order to identify HSPs by western blotting (Towbin et al., 1976). For western blot analysis, primary and secondary antibodies were used to detect sHSP. Primary antibody raised in rabbit for small HSPs (15-30Kd) was graciously provided by Dr Scott Heckathorn, Syracuse University, New York. Secondary antibodies conjugated with horseradish peroxidase (HRP) (Stressgen Bioreagents Corp, British Columbia, Canada) were used at 1:3000 dilutions to detect primary antibodies. Membranes were stained for HRP activity. Incubating the membrane in HRP color reagent provided the color reaction.

Experimental Design and Statistical Analysis

Plants were arranged in a completely randomized design inside each growth chamber/ treatment with six replicate plants per cultivar. Statistical analysis was

performed using SAS (Statistical Analysis Software, version 9.0, Cary, NC). A variance analysis, using ProcReg procedure of SAS was performed to obtain r^2 for sigmoidal curve.

RESULTS

Membrane Damage

Gaillardia and Coreopsis: Membrane damage with increasing incubation temperature, increased for both coreopsis and gaillardia (Fig. 3.1). Percent membrane damage increased more rapidly starting at 45 °C for both the plant species. A shift in the CMT response curve or LT_{50} between the two plant species however, showed no significant difference.

F1 Nature and F1 Iona: A similar sigmoidal response of membrane damage due to increasing temperature was found in the two pansy cultivars (Fig. 3.2). Shift in the CMT response curve or *LT50* showed no significant difference between the two cultivars of pansy studied.

Vista and Sizzler: With increasing temperature treatment percent membrane damage increased for Vista and Sizzler. A significant shift in CMT response curve was observed in 'Sizzler Red', due to greater damage to the membrane or electrolyte leakage when compared to 'Vista Red' (Fig. 3.3). 'Vista Purple' and Sizzler Purple showed similar results (data not shown).

Western Blotting

Gaillardia and Coreopsis: Western blot analysis of protein extract from both heat stressed and control plants of Gaillardia and Coreopsis leaf and stem samples showed no cross reactivity with the antibodies used for detection of small heat shock proteins (Data not shown).

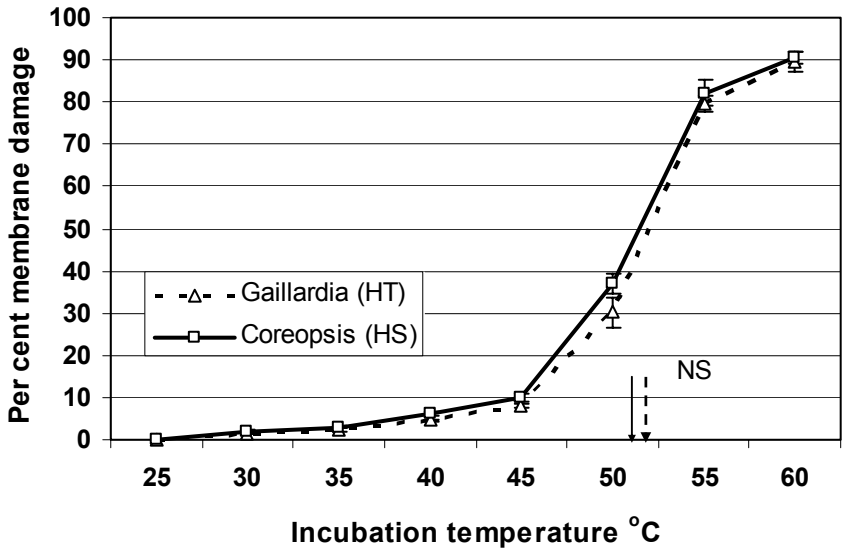

Figure 3.1. Lethal temperature for fifty percent membrane damage (LT_{50}) of leaves in Gaillardia (*Gaillardia x grandiflora*) 'Goblin' series and Coreopsis (*Coreopsis grandiflora)* 'Sunray' series grown at 25 °C. The LT_{50} values are indicated with arrows for each cultivar. Error bars represents standard error. Sigmoidal fit r^2 for Gaillardia= 0.59, r^2 for Coreopsis= 0.48.

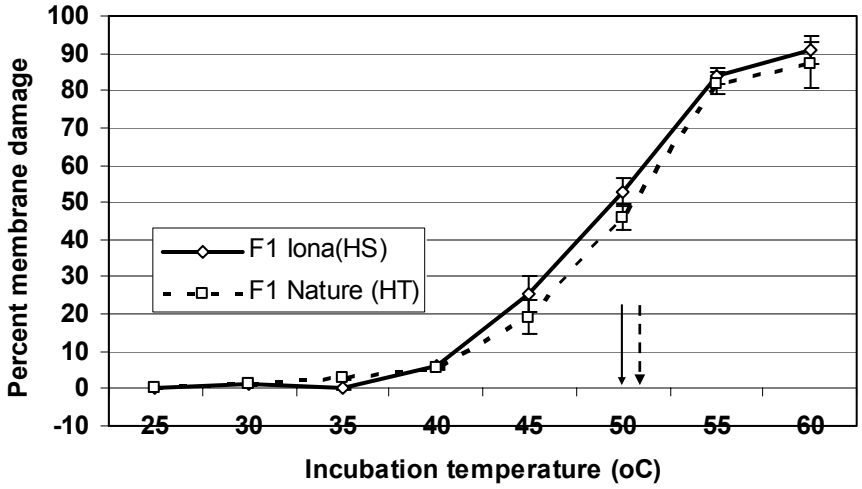

Figure 3.2. Lethal temperature for fifty percent membrane damage (LT_{50}) of leaves in pansy (*Viola x wittrockiana*) 'F1 Nature' series and 'F1 Iona' series grown at 25 °C. The LT_{50} values are indicated with arrows for each cultivar. Error bars represents standard error. Sigmoidal fit r^2 for 'F1 Nature'= 0.68, r^2 for 'F1 Iona'= 0.67.

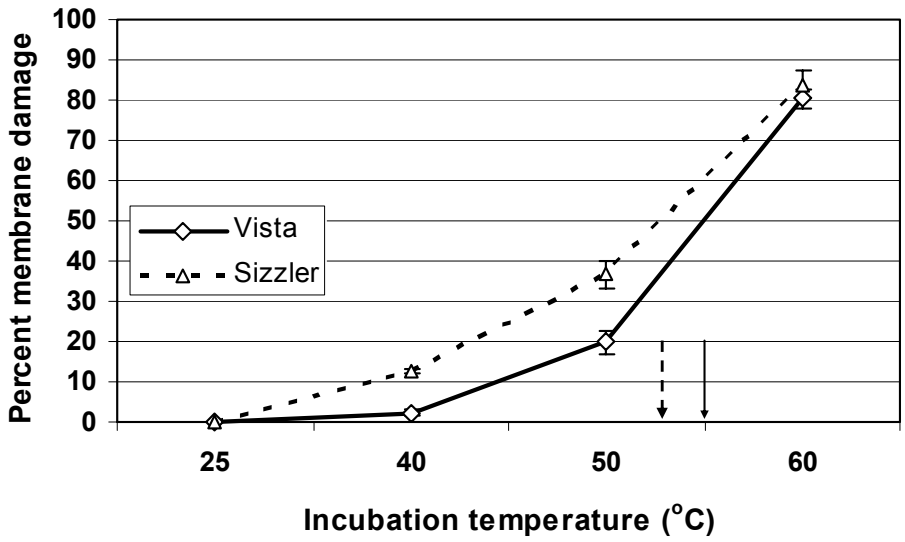

Figure 3.3. Lethal temperature for fifty percent membrane damage (LT_{50}) of leaves in salvia (*Salvia splendens*) 'Vista' series and 'Sizzler' series grown at 25 °C. The LT_{50} values are indicated with arrows. Error bars represents standard error. Sigmoidal fit r^2 for Vista= 0.66, r^2 for Sizzler= 0.76.

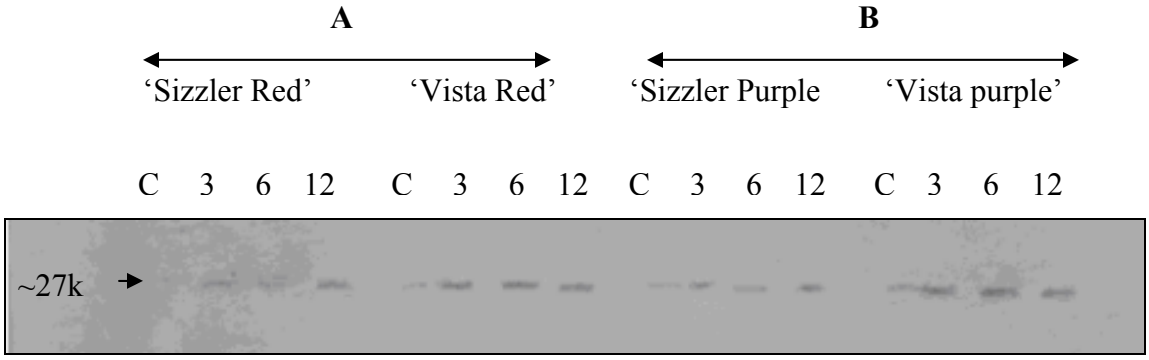

Figure 3.4. Western blot analysis of leaf samples of *Salvia splendens* A) 'Sizzler Red' and 'Vista Red' B) 'Sizzler Purple' and 'Vista Purple', when exposed to 40 °C for control (C), 3, 6 or 12 h.

Pansy F 1 ‘Nature’ and F 1 ‘Iona’ series: Leaf protein extracts from leaves and stem tissue of both the F1 Nature and F1 Iona showed no cross reactivity with the antibodies for small heat shock proteins. Leaf samples of F1 Iona however, resulted in some bands of higher molecular weight (more than 50 kD) more distinctly in leaf samples (data not shown).

Salvia ‘Sizzler Red’ and ‘Vista Red’: Protein extract from the leaf samples of heat stressed plants of both the series of salvia showed bands of high and low molecular weights, however low molecular weight bands of approximately 27kD are more distinct. (Fig. 3.4). Stem and root samples showed no cross reactivity with the antibodies used for detection of heat shock proteins (data not shown).

DISCUSSION

Results from this study identified heat tolerance in some of the cultivars based on membrane stability and sHSP synthesis. Gaillardia and Coreopsis showed no significant difference in shift of CMT response curves or LT_{50} values. These two plant species however, in earlier studies showed differences in growth as defined by shoot and root dry weight, gas exchange measurements and soluble carbohydrate analysis when grown under supra-optimal temperature stress (Vige, 2001).

Pansy cultivars F1 ‘Iona’ and F1 ‘Nature’ showed increasing membrane damage at 45 and 50 °C incubation, however there was no difference in response between the the cultivars. The growth pattern of ‘Nature’ showed some heat tolerant traits, such as smaller leaves and flowers, highly branched, denser looking plant than ‘Iona’ (personal observation). For salvia cultivars Vista and Sizzler, there was a difference of 3.8 °C between ‘Vista Red’ and ‘Sizzler Red’ and 4 °C between ‘Vista’ Purple’ and ‘Sizzler Purple’. For both the red and purple colored cultivars the LT_{50} for Vista series was higher

than the Sizzler series which implies that the leaf tissue of Vista are more heat tolerant than Sizzler. Similar observations were reporter in earlier studies, for example the membrane damage determined in leaves of two leguminous plants *Prosopis chilensis* (a tree species) had a LT_{50} of 6 °C higher than herabaceous soybean (*Glycine max*) (Ortiz and Cardemil, 2001). Greater LT_{50} values imply that the foliar tissues in these plants are more heat tolerant, similar to reports on soybean (*Glycine max*) and *Phaseolous* (Martineau et al., 1979; Schaff et al., 1987).

The western blot analysis for heat shock proteins of these bedding plants indicated variable results. Leaf samples of gaillardia and coreopsis had protein bands of approximately 60 to 70 kD for both control (25 °C) and heat treated.These may be a combination of constitutive and inducible heat shock proteins. Two forms of HSP70 proteins were reported in *Prosospis chilensis* (Medina and Cardemil, 1993), one inducible of 71kD and another constitutive of 69kD (Ortiz et al., 1995). Antibodies for lower molecular weight HSPs poorly cross reacted in coreopsis and gaillardia leaf samples and no distinct bands were noticed. A possible explanation could be the absence of specific antibodies or lower concentrations of proteins in the sample. Similarly, cross reactivity of protein samples of the two pansies were poor and no distinct bands of small HSP were found, however they were slightly detected to a greater extent in ‘Nature’ series than the ‘Iona’ series, possibly due to higher thermotolerance nature over ‘Iona’ series.

Small HSP expressions were distinct in the case of salvia leaf samples compared to stem and root samples. In all the leaf samples of salvia both heat sensitive and heat tolerant HSPs of approximately 27kD were detected. The protein bands however were more qualitatively distinct in ‘Vista’ series of both red and purple cultivars compared to

'Sizzler'. Similar results were observed in several crop plants. For example two different lines of creeping bent grass (*Agrostis palustris* Huds) differing in heat tolerance subjected to 40 °C heat stress treatment significantly differed in HSP25 expression (Park et al., 1996). Colombo et al. (1992) reported a relative difference in HSP synthesis in heat tolerant and heat sensitive clones of black spruce (*Picea mariana*), with heat tolerant groups synthesizing higher constitutive levels of HSPs compared to heat sensitive clones.

Based on these preliminary experiments additional experiments were conducted on high temperature stress responses of salvia 'Vista Red' and 'Sizzler Red'. Pansy cultivars used in this study however, resulted no useful data for subsequent experiments. Hence two other cultivars, 'Crystal Bowl Purple' and 'Majestic Giant Red' were selected based on the plant growth and development and gas exchange results obtained by Erwin et al. (2003).

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CHAPTER 4. STUDY OF HEAT SHOCK RESPONSES OF SALVIA (*SALVIA SPLENDENS*) FOR SCREENING HEAT TOLERANT PLANTS

INTRODUCTION

High temperature stress is one of the primary abiotic factors which influence the growth and development of many plants during the late spring and summer in most parts of the world (Koh, 2002). Extreme high temperature often leads to several closely related abiotic stresses such as water deficit and drought stress influencing many biochemical and physiological responses in plants. Heat injury in plants, as defined by Levit (1980), results from short exposure within seconds to 30 min to extreme temperatures of 45 to 65 °C. In supraoptimal temperatures, soil temperatures often reach injuriously high levels affecting root growth, strongly influencing shoot growth, leaf senescence and survival of whole plants (Aldous and Kaufmann, 1979; Kuroyanagi and Paulsen, 1988; Paulsen, 1994; Udomprasert et al., 1995; Xu and Huang, 2004). The most common visible symptoms of heat stress are wilting of leaves, marginal foliar burning, necrosis of leaf surfaces and poor flowering (Hall, 2001). In order to avoid such injuries, most plants growing in dry regions exhibit adaptive morphological features such as thick leaves, reflective leaf hairs, succulent stems, thick cuticle, and sunken stomata (Beadle, 1981), higher membrane thermostability (Yeh and Hsu, 2004), higher stomatal conductance, transpiration and net photosynthesis (Lu et al., 1997) and greater production of heat shock proteins (Downs et al., 1998; Knight and Ackerly, 2003).

Several studies indicated that leaf injury under supraoptimal temperatures are due to direct inhibition of root growth, hormonal perturbation and its transport, water and nutrient uptake (Graves et al., 1991; Huang et al., 1991; Kramer, 1983; Gur and Shulman,

1979; Huang and Xu, 2000; Huang et al., 2001). Physiological effects include increased respiration rate, reduced net photosynthesis, loss of cell water content and turgidity leading to closure of stomata. Guilioni et al. (1997) reported that a short period of heat stress could cause a siginificant rate of floral bud abortion in pea (*Pisum sativum*), tomato (*Lycopersicum esculentum*) (Levy et al., 1978), snap bean (*Phaselous vulagris*) (Konsens et al., 1991), and cotton (*Gossypium* spp) (Reddy et al., 1992).

Another signature physiological response of plants to heat stress is decreased synthesis of normal proteins accompanied by an accelerated increase of a group of proteins with a molecular mass of 15 to 42 kD, designated as small heat shock proteins (sHSP) (Hsieh et al., 1992; Wehmer and Vierling, 2000; Lohmann et al., 2004). Plants are characterized by unusually abundant and diverse sHSP that may reflect their need to quickly adapt to ever changing environmental conditions such as temperature, light, and humidity (Sun et al., 2002). Small heat shock proteins act as molecular chaperones and protect biomolecules from detrimental effects of heat stress; increased levels of sHSP are positively correlated with heat tolerance (Clarke and Critchley, 1994; Heckathorn et al., 1996; Basha et al., 2004). Most research on high temperature stress responses has been studied on agronomic and turfgrass species with little emphasis on herbaceous ornamental annuals. Therefore, the study of ornamental bedding plants that experience severe heat stress in landscapes after transplanting and during transition period is warranted.

Lasseigne et al., (1998) studied heat tolerance based on plant performance of different taxa of salvia such as European species that prefer cool and moist conditions and Mexican species adapted to hot sunny sites. European species at 35 °C and 40 °C resulted in greater amounts of damage in terms of general stunting, distortion and

necrosis of leaf blade than Mexican species. Major morphological and physiological characteristics associated with better heat tolerance of many newly developed ornamental salvia and other popular bedding plants, have not been studied. Understanding the relative involvement of various morphological and physiological characteristics in heat tolerance of salvia would help to identify traits of heat tolerance and facilitate breeding programs in developing heat tolerant cultivars.

Understanding morphological and physiological responses of bedding plant series and/or cultivars differing in heat tolerance would help to better identify the factors responsible for heat tolerance/sensitivity. Therefore, this experiment was designed to 1) study the morphological characteristics such as plant growth, shoot and root dry matter accumulation, overall marketable quality, and 2) study the physiological characteristics such as transpiration, stomatal conductance, net photosynthesis, percent membrane damage, and small HSP synthesis in response to heat stress of two salvia cultivars 'Vista Red' and 'Sizzler Red' differing in heat tolerance.

MATERIALS AND METHODS

Plant Materials

Two cultivars of *Salvia splendens*, 'Vista Red' referred as Vista and 'Sizzler Red' referred as Sizzler, were selected for use in this study based on their ability to grow at high temperatures as described by Pan American Seed (personal correspondence). Vista flower later than Sizzler allowing more time for establishment and have a darker green foliage providing for a healthier looking plant. Vista tend to grow and flower better at higher temperatures than Sizzler. Seeds of Vista and Sizzler (Pan American seed Inc.). Seeds were germinated in 10cm pots filled with Jiffy Mix® (Jiffy Products, Batavia, IL) in environmental growth chambers (EGC, Chagrin Falls, OH). Growth chambers were

programmed to maintain 25/18 °C day/night cycles with a 14 h photoperiod of 500 μmoles m^{-2} s^{-1} PPFD (photosynthetic photon flux density). Plants in the growth chambers were fertigated every morning at approximately 0900H with 100 mg/L 15N-2.2P-12.4K (15-5-15 Cal Mg, Scotts-Sierra, Marysville, OH).

Temperature Treatments

Three week old plants were subjected to four short term high temperature treatments for 3 h on every third day in growth chambers. Temperature was gradually ramped up in 5 °C increments every 30 min from 25 °C to 30, 35, 40 and 45 °C. These high temperature treatments were maintained for 3 h and then ramped down similarly to 25 °C. These temperature treatments were based on similar studies using other crops (Crafts-Brandner and Salvucii, 2002) with some modifications. Temperature treatments were continued until completion of flowering (approximately 10 weeks) in the growth chambers to study the morphological responses of plants to short duration high temperature stress.

Plant Growth and Development

Plant height was recorded from soil line to the apical meristem and measured on the individual plants. Internode length and stem thickness were measured at the third internode from apex. Stem thickness were determined using a digital calipers (VWR Scientific Instruments, Suwanee, GA) and averaging two measurements that were taken one perpendicular to the other. All the plants were destructively harvested after flowering and total leaf area per plant was measured using LI-3100C leaf area meter (LI-COR Biosciences, Lincoln, NE). Shoot and root dry weights were obtained after oven drying at 80 °C for 24h.

Gas Exchange Measurements

Physiological parameters such as transpiration rate (T), stomatal conductance (gs), and net photosynthesis (Pn) were measured on two recently matured leaves using CIRAS-I portable photosynthesis system (PP Systems, Amesbury, MA) 24 h after a heat stress treatment. Two readings per leaf were taken under artificial light source providing equal light intensity of 500 μmoles m^{-2} s^{-1} as that of growth chambers used in this experiment.

Electrolyte Leakage

Electrical conductivity of electrolytes was measured according to the method previously described (Raymond et al., 1986; Hallam and Tibbits, 1988) with some modifications. Eight week old plants grown at 25/18 °C were acclimatized for 1 day at 35/28 °C in the growth chamber. Five mm diameter leaf discs were punched from fully matured leaves on either side of the mid rib using a cork borer. Freshly collected leaf discs were washed twice with distilled deionized water in a test tube and after draining the water; test tubes were sealed with aluminum foil and incubated in thermostat controlled water baths set at 30, 35 40 45, 50, 55 and 60 °C for 1 h. After tissue incubation, the test tubes were cooled to room temperature and 25 ml deionized water were added to the tubes for incubation at 25 °C for 1 h, under agitation. Conductivity of water surrounding the leaf tissue was measured using a conductivity meter (VWR Scientific Instruments, Suwanee, GA). For measuring maximum membrane damage, test tubes with leaf discs were autoclaved for 15 min at 0.1Mpa and conductivity of water was recorded. Electrical conductivity measurements were used to calculate lethal temperature (LT_{50}). LT_{50} is defined as the temperature at which there is 50% membrane damage determined by the conductivity of water surrounding the tissues treated at different

increasing temperatures (Ortiz and Cardemil, 2001). The following equation was used to calculate percent membrane damage:

$$\% \text{ of membrane damage} = \frac{Cx - Cc}{Cm - Cc} \times 100$$

Where Cx is the conductivity of water surrounding the leaf tissue incubated at different temperatures for 1 h, Cc is the conductivity of water of the sample at 25 °C, and Cm is the maximum conductivity after autoclaving the leaf tissue.

Total Water Soluble Sugar Content (WSS)

Leaves harvested after taking CIRAS-I data were immediately frozen in liquid nitrogen and stored at −40 °C until further analysis. Frozen leaf tissue was lyophilized, weighed, and ground to pass a 20 mesh screen. Water soluble sugars (WSS) were extracted and measured using the Miller and Langhans (1989) procedure three times with one milliliter (ml) with 12 methanol: 5 chloroform: 3 water (MCW) by volume. Fifty milligrams of finely ground leaf tissue was weighed into disposable Pasteur pipets fitted with glass wool plugs. One ml of MCW solution was added to each pipets, to rinse the sides of tube another 0.5ml of MCW solution was added and stirred with a glass rod and left to set for one hour. Then the pipets were drained with compressed nitrogen to drain the solution from the pipets to labeled 15 ml centrifuge tube. Mannitol (1.0 mg) was used as an internal standard and added at the beginning of the first MCW extraction. After extraction was complete, each pipet was rinsed with 1 ml MCW. After final drain of solution 3 ml of distilled deionized water was added to partition out the chloroform by centrifugation in a swinging bucket centrifuge for 20 min at 12000 g. After centrifugation the aqueus phase was removed and filtered through polyethylene columns containing 1 ml Amberlite IRA-68, acetate form, and 1 ml Dowex 50-W, hydrogen form (Sigma-

Aldrich, St.Louis, MO) resisns. Columns were washed twice with 0.5 ml Methanol: water (1:1).

The aqueus phase was then vaccum dried using an Evapotech^{TM} (Haake Buchler, Saddle Brooke, NJ) with a refrigerated condensation trap RT100 (Savant Ins. Farmingdale, NY) to concentrate the soluble sugars. To the concentrated sugars 2 ml of HPLC- grade water was added to resuspend and forced through a 0.45µm membrane filter using a 13mm plastic swinney filter holder (Pall, Gelman Lab, Ann Arbor, MI) to disposable vials. Filtered samples were injected into Waters HPLC system (Milford, MA) using HPLC-grade water as mobile phase at the rate of 1.0 ml per minute. Total water soluble sugars (WSS) were separated on a Shodex 1011 column (J M Sciences Inc. Grand Isaland, NY) maintained at 80 °C using a column heater. Total sugars were detected by refractive index and determination of specific sugars was based on the comparison of retention times to those of authentic D-sugars, sucrose, raffinose, glucose and fructose (Sigma # S-9378, # R-0250, #G-5250, and # F-0127, respectively).

Plant Tissue Preparation, SDS PAGE, and Western Blotting

Leaf samples collected and stored after recording CIRAS-1 data were used for protein analysis. Frozen tissue samples from both heat stressed and non heat stressed plants were ground to fine powder using liquid N_2 and extracted using the protocol from Downs et al. (1998) in buffer containing 100 mmol/L Tris-HCl (pH 8.0), 1% sodium dodecyl sulfate (SDS; w/v), 1% dithiothreitol (DTT; w/v), 1 mmol/L phenyl methyl sulfonylfluoride (PMSF), 5 µmol/L leupeptin, 5 mmol/L ε-amino caproic acid, 1% ascorbate (w/v), and 3 mmol/L Na_2EDTA (Sigma Chemical Co., St Louis, MO). Polyvinylpyrrolidone (PVP) 3 % (w/v) and /or 30 mmol/L sodium tetraborate was used to

remove phenolics. Samples were boiled for 3 min and centrifuged at 14000 g for 6 min. The supernatant was collected and stored at -80 °C.

Protein content from leaf extract was determined according to Bradford assay (Bradford, 1976), BSA was used as the standard. The soluble proteins were fractionated on 15 % one dimensional SDS-PAGE gels as described previously by Laemmli (1970) and electro transferred to a 0.45µ nitrocellulose membrane (Towbin et al., 1979). After protein transfer, the nitrocellulose membranes were blocked in 1% (w/v) BSA and incubated with the primary and secondary antibodies. Molecular mass standards were included on all gels (Precision Plus Protein standards, BioRad, Hercules, CA). The relative difference in protein-antibody complexes were estimated by densitometry using a desktop scanner (Scanjet 3300C, Hewlett Packard, Palo Alto, CA) and ImageJ imaging software (ver1.33) (http://rsbweb.nih.gov/ij/).

Marketable Quality

Overall marketable quality of plants was assessed based on 1 to 10 scale with 10 being the best and 1 being the worst. Marketable quality scores; 9 and 10= excellent plants with healthy green leaves and good inflorescence, 8= green healthy foliage with moderate flowers, 7= plants with poor inflorescence, 5 and 6= necrotic leaves and poor flower set, 4= terminal bud damage, 2 and 3 = dried leaves and 1 = dead.

Experimental Design and Statistical Analysis

Plants were arranged in a completely randomized design inside each growth chamber/ treatment with six replicate plants per cultivar. Statistical analysis was performed using SAS (Statistical Analysis Software, version 9.0, Cary, NC). A variance analysis, using ProcMix procedure of SAS was performed and significance of differences in mean was determined by Tukey's test.

RESULTS

Plant Growth and Development

High temperature treatment of 45 °C severely affected seedlings of both the cultivars during the early stages, and all the seedlings died by the commencement of the experiment. Periodic short duration high temperature treatments resulted in a decrease in plant height, stem thickness and total leaf area per plant for Sizzler compared to control. A short duration exposure of 30 °C and 35 °C for 3 h every third day did not affect plant height in either of the series (data not shown). Exposure of plants to 40 °C reduced the plant height in both the series. Percent reduction in plant height was much greater for Sizzler (34.58 %) compared to Vista (12.54 %) at 40 °C (Fig 4.1).

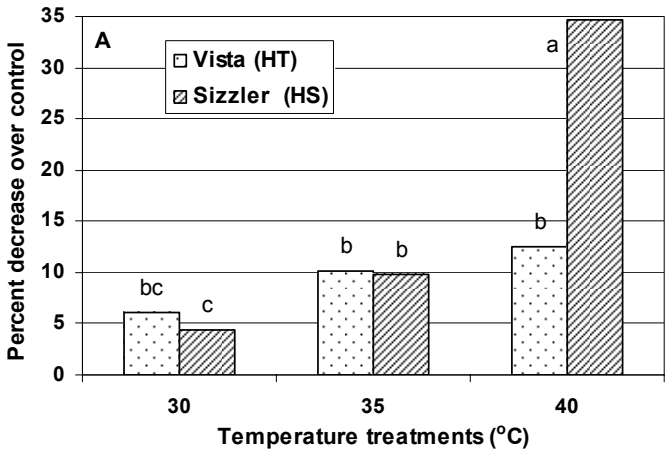

Figure 4.1. Effect of temperature treatment for 3h every third day on plant height in *S.splendens* Vista (heat tolerant) and Sizzler (heat sensitive). Means with different letters are significantly different at $P<0.05$ (Tukey's test).

As temperature treatment increased stem thickness decreased for both the cultivars (Fig 4.2). Vista had significantly thicker stems compared to Sizzler at control and high temperature treatments. The dry weights of plants recorded at the end of the experiment are also affected by high temperature treatments. With increase in

temperature treatment both root and shoot dry matter accumulation decreased for both cultivars (Fig 4.3).

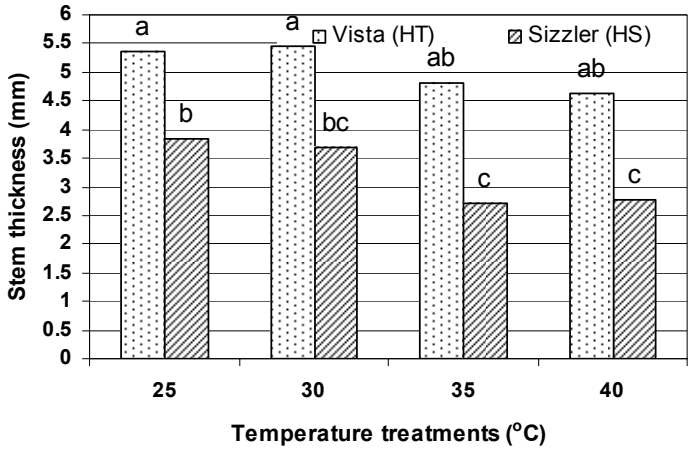

Figure 4 2. Effect of temperature treatment for 3h every third day on stem thickness in *S.splendens* 'Vista Red' (heat tolerant) and 'Sizzler Red' (heat sensitive). Means with different letters are significantly different at P<0.05 (Tukey's test).

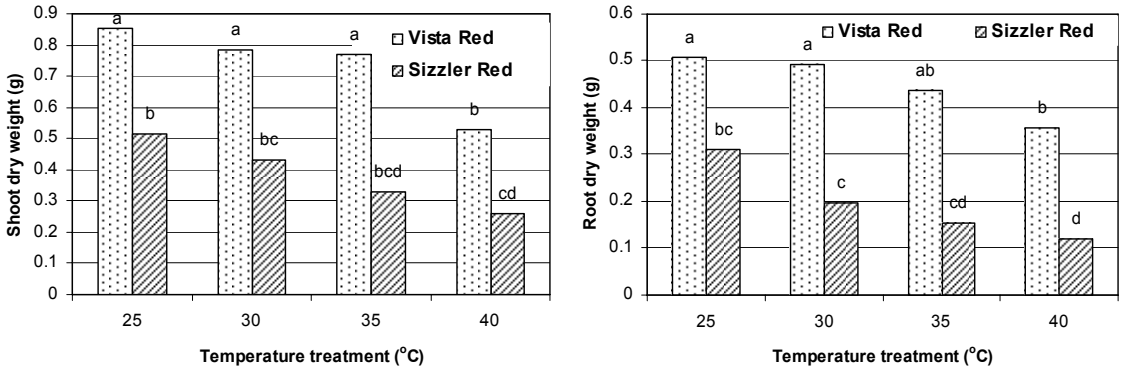

Figure 4.3. Effect of temperature treatment for 3h every third day on shoot and root dry weights in *Salvia splendens* cultivars 'Vista Red' (heat tolerant) and 'Sizzler Red' (heat sensitive). Means with different letters are significantly different at P<0.05 (Tukey's test).

At all temperature treatments Vista maintained greater plant dry weight compared to Sizzler. Root to shoot ratio (R:S) results also showed that, as temperature treatment increased R:S decreased in Sizzler while Vista maintained greater R:S values even at the 40 °C temperature treatment (4.4)

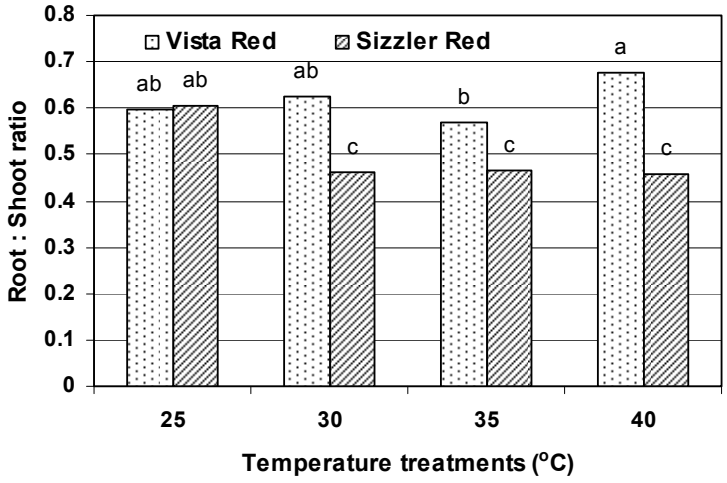

Figure 4.4. Effect of temperature treatment for 3h every third day on root to shoot ratio in *S.splendens* cultivars 'Vista Red' (heat tolerant) and 'Sizzler Red' (heat sensitive). Means with different letters are significantly different at $P<0.05$ (Tukey's test).

Transpiration (T), Stomatal conductance (Gs) and Net photosynthesis (Pn)

As temperature treatment increased above 30 °C all three parameters measured increased and beyond 35 °C these parameters declined (Fig.4.5). Vista maintained significantly greater T, Gs, and Pn at 35 and 40 °C compared to control and Sizzler plants. At 45 °C treatment all the parameters measured were below control levels for both the cultivars.

Total Soluble Sugars

Analysis of total water soluble sugars (WSS) showed raffinose and sucrose as the primary sugars in salvia leaves (Fig 4.6A & B). In Vista the maximum concentrations of raffinose and sucrose was observed at 35 °C treatment. Beyond this temperature treatment raffinose concentrations decreased. Sucrose levels increased as temperature treatment increased for both the cultivars tested (Fig. 4.6B). Glucose and fructose concentrations remained relatively constant for Sizzler over all temperature treatments (Fig 4.6C & D).

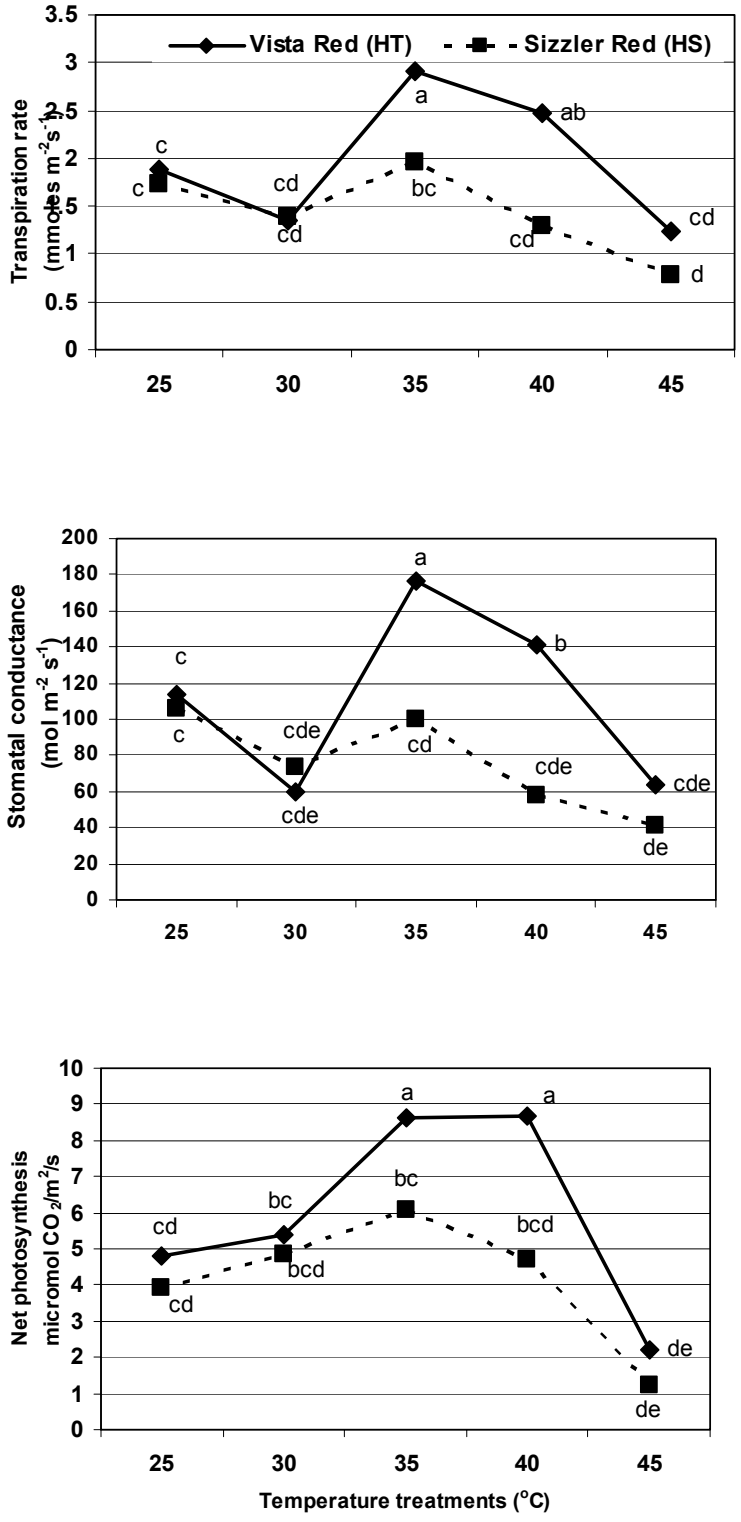

Figure 4.5 Effect of temperature treatment for 3h every third day on transpiration, stomatal conductance and net photosynthesis in two cultivars of *S.splendens* 'Vista Red' and 'Sizzler Red'. Asterix represents significant difference at P<0.05 (Tukey's test).

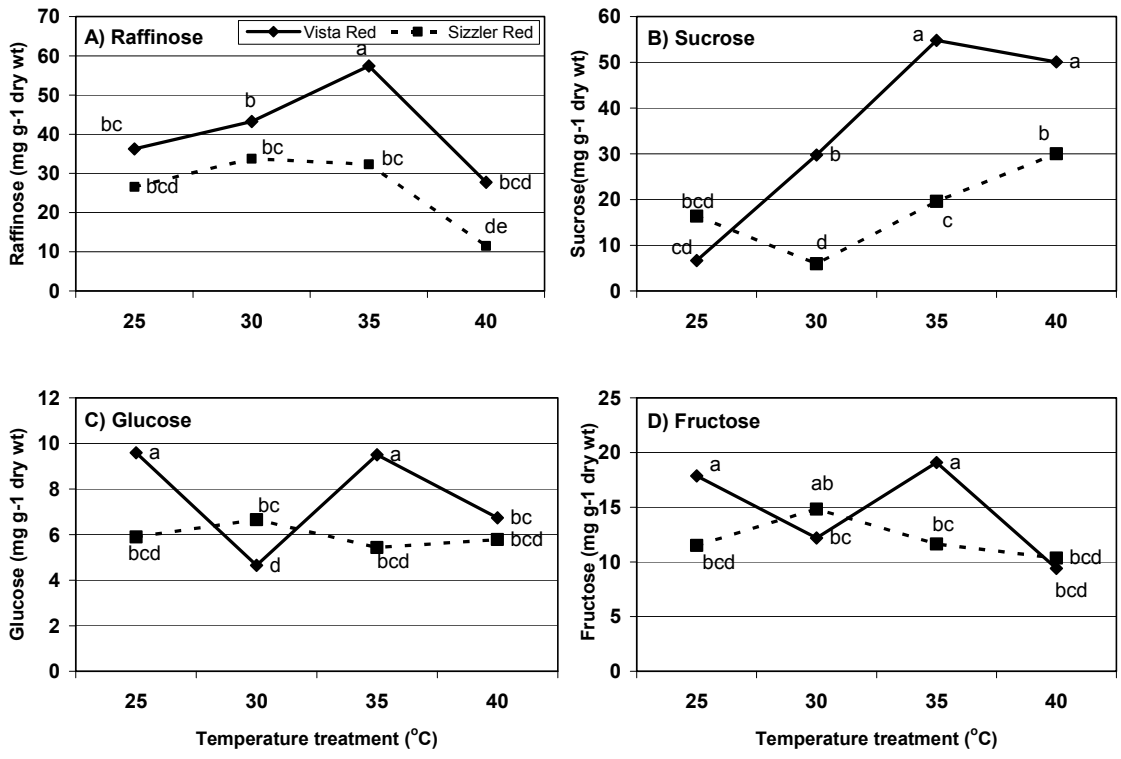

Figure 4.6. Effect of temperature treatment for 3h every third day on soluble sugars concentrations in leaves of *S.splendens* cultivars 'Vista Red' (heat tolerant) and 'Sizzler Red' (heat sensitive). Means with different letters are significantly different at $P<0.05$ (Tukey's test).

Electrolyte Leakage

Percent membrane damage increased in a sigmoidal fashion; a slower rate initially from 25 °C to 50 °C and as incubation temperature increased, conductivity increased very rapidly between 50 and 55 °C (Fig 4.7). Maximum membrane damage occurred at above 55 °C. Membrane damage was greater for Sizzler compared to Vista at 40, 45 and 50 °C.

Western Blotting

As the temperature treatments increased from control (25 °C) to the highest temperature treatment (45 °C), the production of small HSP of approximately 27kD increased for both the cultivars. For Vista, distinct protein bands were observed starting

at 30 °C and intensity of these bands increased as treatment temperature increased (Fig. 4.8C). While in Sizzler bands were only identified starting at 40 °C. At 45 °C both the cultivars resulted in similar expression levels of HSP27 (Fig. 4.8A & B).

Marketable Quality

On a scale of 1 to 10, a score of 7 or above was considered as acceptable marketable quality. Vista showed no severe symptoms of leaf damage or flower drop even at 35 °C when compared to Sizzler. Marketable quality began to decline at 30 °C. Sizzler were more susceptible to higher temperature exposure than Vista as marketable quality decreased at a greater rate from 30 to 40 °C (Fig 4.9). Severe necrosis of leaves was noticed in Sizzler indicating its greater sensitivity to high temperature (Fig.4.10).

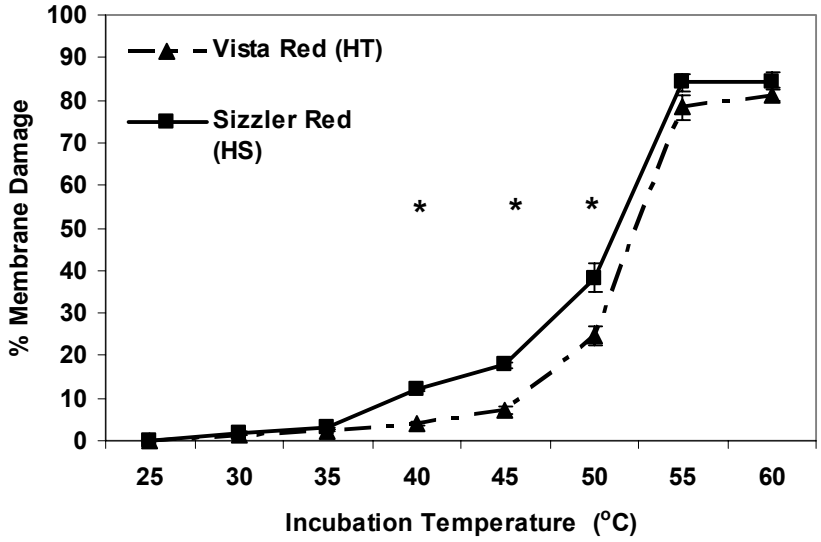

Figure 4.7. Effect of short duration high temperature treatment percent membrane damage in leaf tissue of *Salvia splendens* 'Vista Red'and 'Sizzler Red'. Asterix indicates significant difference at P=0.05. Sigmoidal fit $r^2 = 0.66$ for Sizzler and $r^2 = 0.71$ for Vista.

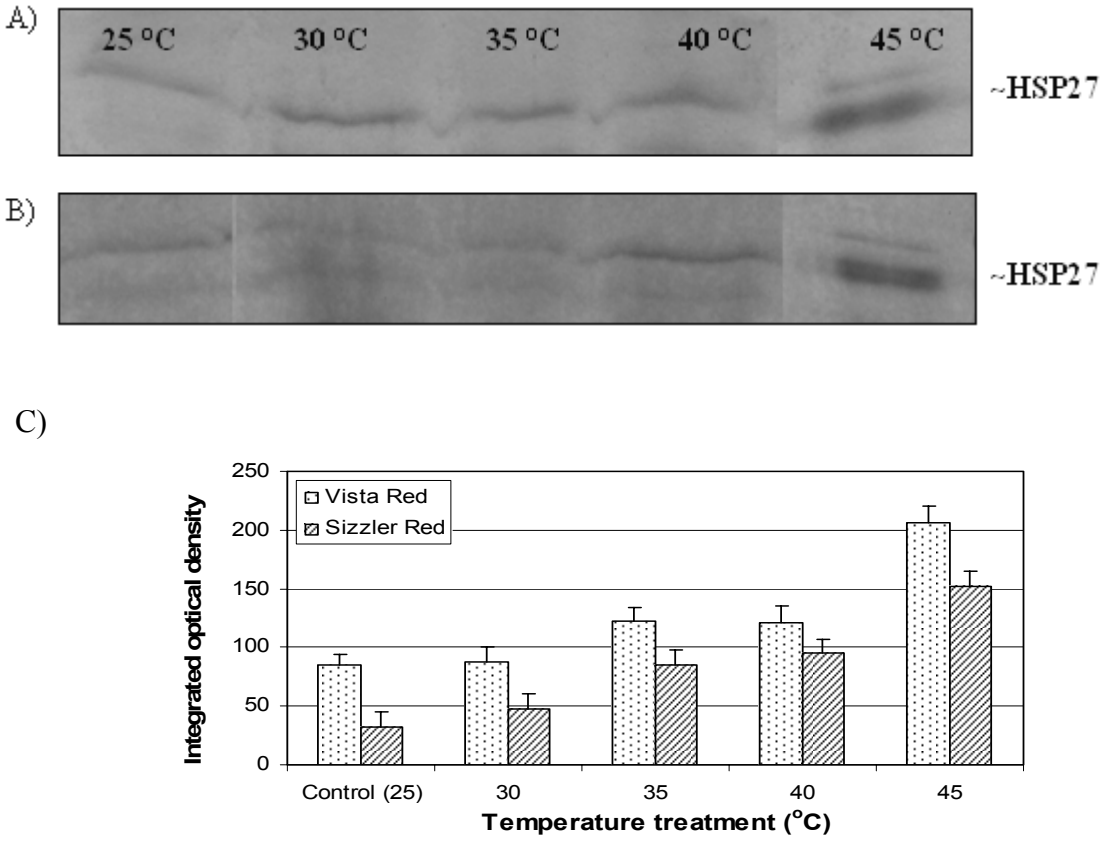

Figure 4.8. Effect of temperature treatments applied for 3h on accumulation of small heat shock protein 27kD in *Salvia splendens* A) 'Vista Red' and B)'Sizzler Red' C) Integrated optical density values of bands measured with imajeJ software. Error bars represent mean of six measurements ± standard error

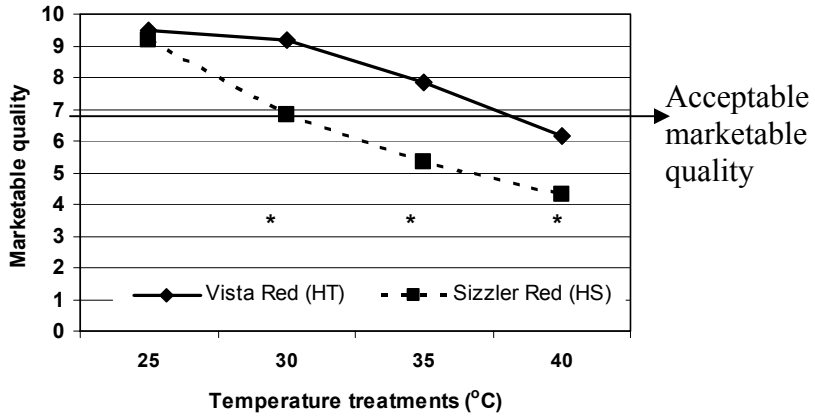

Figure 4.9. Effect of temperature treatments applied for 3h on marketable quality in *S.splendens* 'Vista Red' (heat tolerant) and 'Sizzler Red' (heat sensitive). Asterix indicates significant different at $P<0.05$ (Tukey's test). Quality score of 1= poor and 10= best.

Figure 4.10: Marginal burning symptoms on *Salvia splendens* 'Sizzler Red' leaves of different ages subjected to short duration high temperature stress of 35 °C for 3h every third day.

DISCUSSION

This study indicated that growth of both the cultivars of salvia declined with short duration high temperature stress treatments. Vista however showed a lesser degree of damage when compared to Sizzler. Heat stress beyond certain specific temperature is unfavorable for growth of many plants because of accelerated increase in transpiration water loss, reduced stomatal conductance, net photosynthesis (Griffin et al., 2004). This critical temperature differs from species to species of plants (Law and Crafts-Brandner, 1999) and therefore it is difficult to define one critical temperature for all plants. In this study the critical temperature for salvia was between 30 and 35 °C.

Net photosynthesis of Vista and Sizzler began to decline above 35 °C, a similar pattern was observed for stomatal conductance and transpiration rate. Growths of plants are suppressed beyond this temperature irrespective of heat tolerance levels of these cultivars. Elevated high temperatures increased respiration and therefore require greater carbon fixation for sustatined growth and survival in spinach (*Chenopodium album*) (Brooks and Farquhar 1985). Photosynthesis may also be limited at temperatures above 35 °C with decreased Rubisco activity as was found in tobacco (*Nicotiana* species) and

cotton (*Gossypium* species) (Crafts-Brandner and Salvucci, 2000). Hence the capacity of plants to acclimate and maintain photosynthesis under high temperature is a critical factor in heat tolerance (Hale and Orcutt, 1987).

A possible explanation for decline in WSS in leaf tissue of Vista and Sizzler could be due to reduced rates of net photosynthesis beyond 35 and 30 °C as was also found in cotton (*Gossypium hirsutum* L) and wheat (*Triticum aestivum* L.) respectively (Feller et al., 1998). Maximum amounts of WSS were observed at 35 °C temperature treatment for heat tolerant cultivar Vista (Fig. 4.6). Sucrose concentration for Vista however increased as temperature treatment increased (Fig.4.6B).This could be due to an increase in sucrose recycling under high temperature and reduced sucrose degradation as was found in potato (*Solanum tuberosum*) (Geigenberger et al., 1998). Jiao and Grodzinski (1996) reported increased sucrose and raffinose accumulation in leaves of *S.spledens* at 40 °C. They indicated that an increase in raffinose and sucrose in another heat tolerant salvia was associated with the osmolyte accumulation which increased heat tolerance. Similar conclusions were derived from research with transgenic *Arabidopsis* study, where certain stress inducible enzymes played a key role in accumulation of raffinose and other sugars to function as osmoprotectants under abiotic stress conditions (Taji et al., 2002).

Heat tolerant Vista studied in this experiment showed greater thermostability in terms of reduced percent of membrane damage when compared to heat susceptible Sizzler (Fig 4.7). Greater cell membrane thermostability equates to less membrane damage, providing for healthier leaves and increased shoot dry weights in English ivy (*Hedera helix*) (Yeh and Hsu, 2004). Similar results were found in this study. Membrane thermostability is thought to be associated with phase changes in the lipid bilayer (Suss and Yordanov, 1986). Since photosynthetic and mitochondrial activity depends on

stability of membrane, any amount of membrane disruption under heat stress will have a tremendous impact on their metabolic function (Schreiber et al., 1998). The less damage to the photosynthesizing tissue, greater the net photosynthetic capacity and resulting in assimilation of food reserves. Heat tolerant Vista had greater membrane stability, greater net photosynthesis, and greater shoot and root growth than Sizzler. Another possibility of maintenance of greater photosynthetic rates in heat tolerant cultivars could be due to protection of PSII a heat sensitive protein complex by sHSP under high temperature conditions (Heckathorn et al., 2002).

Higher plants produces at least 20 and some plant species produce as many as 40 different small heat shock proteins with molecular weights ranging from 15 to 42 kD (Vierling 1991). Small heat shock protein identified in this study was approximately 27kD, the intensity of bands showed that the HSP27 expression of heat tolerant cultivar Vista was significantly greater than heat sensitive cultivar Sizzler at high temperature treatments (Fig. 4.8C). Similar findings from a study on chloroplast sHSP by Heckathorn et al. (2002) reported a significant differential expression pattern in genotypes of *Agrostis stolonifer* differing in heat tolerance. The possible role of HSPs for heat tolerance in arabidopsis, several agronomic crops and turf grass species were presented in previous research (DiMascio et al.1994, Queitsch et al., 2000, Luo Jinn 2004). However contrasting views related to the role of sHSP and thermotolerance of plants were also expressed by few studies (Park et al., 1996 and 1997). Authors stated that expression of a mere one to three additional HSPs from a multigene family might *not* likely result in increased thermal tolerance. From this study, however sHSP and the morphological and physiological parameters presented and discussed clearly indicated relationship among

some of the measurable heat tolerant traits and the overall marketable quality of finished plants.

This is the first report showing that morphological, physiological and sHSP measurements following heat shock can be used to predict whole-plant heat tolerance among bedding plant species. The results from this study indicate that heat tolerant cultivar Vista possesses three mechanisms helps to cope with heat stress a) morphological traits of short stature, thick stems and leaves b) greater membrane stability, greater Gs, T and Pn. and c) increased synthesis of sHSP at high temperature. Sizzler showed high sensitivity to heat stress, however it appeared to have a high potential to acclimate to moderately high temperature. Hence in subsequent studies short duration heat exposure will be used as a "heat preconditioning" treatment to test induction of heat tolerance in heat sensitive as well as heat tolerant cultivars. These results have also laid the foundation for a laboratory screening technique, to test heat tolerance of bedding plants. Currently the only available selection procedures for landscape plants include regional field trials (Albrecht and Pair, 1994). In addition to taking many years for selecting superior cultivars, this method is largely inefficient or not quantitative because of unpredictable heat stress events and interactions with other environmental factors. Levitt (1980) reported that various abiotic environmental factotrs interact with heat stress, such as water status/stress, soil nitrogen levels (Wehner and Watschke, 1981), previous growing temperature (Wehner et al., 1985), relative humidity (Perdomo et al., 1996). Chen et al., (1982) reported that in field studies the rate of change and the duration and magnitude of high temperatures contribute to the intensity of crop plant heat stress. Thus future research will focus on 'heat preconditioning' and development of an accurate but simple heat tolerance test.

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CHAPTER 5. SHORT DURATION HEAT PRECONDITIONING AND HEAT TOLERANCE IN SALVIA (*SALVIA SPLENDENS*)

INTRODUCTION

High temperature is one of the primary abiotic stress affecting plant growth and development in most parts of the world (Leong S.K., 1983). Supraoptimal temperature (10-15 °C above normal temperature, 25 °C) increases respiration and therefore requires greater CO_2 fixation for sustained growth and survival. Furthermore, given the present trend of global warming, temperatures are likely to become even hotter (Houghton et al., 1990). Air temperatures greater than 35 °C significantly decrease Rubisco activity thereby limiting photosynthesis in cotton (*Gossypium hirsutum* cv Coker 100A glandless) and tobacco (*Nicotiana rusticum* cv Pumilla) (Crafts-Brandner and Law, 2000). Genotypes or cultivar lines within species differ in their ability to respond to heat stress conditions. For example 12 open pollinated families of white spruce (*Picea galuca*) seedlings differing in growth performance when exposed to high temperatures of 42 to 50 °C for 30 min differed in their extent of damage in terms of chlorophyll fluorescence (Bigras, 2000). Therefore it is important to identify heat-tolerant cultivars for use in breeding programs (Prasad et al., 1999).

To overcome the effects of high temperature, plants also have evolved with remarkable ability to adapt/acclimate and survive in stressful environmental situations (Senthil et al., 2003). One of the primary plant adaptations to tolerate high temperature stress is commonly referred as acquired thermotolerance. Several studies with a number of organisms including plants indicated that a brief exposure to sublethal high temperatures improves the ability of the organism to survive subsequent exposures to potentially lethal temperatures (Levins, 1969 ; Vierling, 1991; O'Connell, 1994; Krebs

and Loeschcke, 1994). Many plants have developed a wide range of adaptable features to high temperatures including morphological (smaller and narrower leaves, spines, reflective trichomes on upper leaf surface, deeper root system), physiological (high transpiration and stomatal conductance) (Thuiller.W, 2003; Xu and Huang, 2001) and anatomical traits (thick cuticle, thicker leaves, increased stomatal density) (Ristic, 1991). The capacity of plants to acclimate and survive under high temperature is a critical factor in heat tolerance. This adaptation and/or acclimatization to temperatures is considered as one of the primary determinants of geographical distribution of plants (Mahan et al., 1997).

Most of the popularly grown bedding plant cultivars released in the market lack some of the previously mentioned characteristics and they fail to survive in high temperatures of landscapes. Understanding morphological, physiological and anatomical adaptations of the whole plant to high temperatures may provide improved production practices prior to planting in the landscapes and selection of traits for breeding programs, leading to development of heat tolerant bedding plant cultivars.

Earlier studies in various plant species including Kentucky bluegrass (*Poa pratensis*. L) (Jiang and Huang, 2001), annual bluegrass (*Poa annua*) (Wehner and Watschke, 1981) and cotton (*Gossypium hirsutum*) (Brown and Thomas, 1980) reported that prior exposure of plants to drought stress (preconditioning) increased subsequent heat tolerance. Preconditioning by frequent soil drying improved heat tolerance in Kentucky blue grass (*Poa pratensis*) under 35/30 °C heat stress in terms of enhanced stomatal conductance and transpiration rate and significantly higher rates of net photosynthesis than non preconditioned plants (Jiang and Huang, 2000). Many of the studies on plant preconditioning in order to improve stress tolerance are focused either on

the combination of heat and drought study or drought stress alone (Jiang and Huang, 2001; Ladjal et al., 2000).

Lasseigne et al. (1998) conducted a heat tolerance study in perennial salvias (*S. sylvestris, S. nemorosa* etc) based on their growth and development under different high temperatures of 20 to 40 °C. They reported that there is considerable variation within different taxa of salvia on severity of heat damage symptoms. Most bedding plants are grown in landscape situations where they encounter severe heat stress during summer months even though they receive ample quantities of irrigation. Although bedding plant production and landscaping have become the most important part of the floral industry; a wholesale value of $2.53 billion the largest contributor to the value of floriculture production (2004 USDA floriculture crop summary). Little work has been done in understanding the heat stress affects and mechanism of adaptation of these plants to high temperature stress. Therefore the objectives of this research were to study heat preconditioning and subsequent heat stress affects on whole plant growth, physiology and leaf anatomy and acclimation in the annual bedding plant salvia (*Salvia splendens*).

MATERIALS AND METHODS

Plant Material

Two cultivars of *Salvia splendens*, 'Vista Red' referred as Vista and 'Sizzler Red' referred as Sizzler, were selected for use in this study based on their ability to grow at high temperatures as described by Pan American Seed (personal correspondence). Vista flower later than Sizzler allowing more time for establishment and have a darker green foliage providing for a healthier looking plant. Vista tend to grow and flower better at higher temperatures than Sizzler. Seeds of Vista and Sizzler (Pan American seed Inc.) were germinated in growth chambers in 10 cm diameter pots using commercial potting

mixture Jiffy Mix® (Jiffy products). Day and night temperatures of 25/18 °C (±1.5 °C) were maintained with 14 h photoperiod of 500 $\mu molem^{-2}s^{-1}$, and a relative humidity of 70 % (±5%). Plants in the growth chambers was fertigated every morning at approximately 0900h with 100 mg/L 15N-2.2P-12.4K (15-5-15 Cal Mg, Scotts-Sierra, Marysville, OH).

Heat Preconditioning and Temperature Treatment

Two week old seedlings after germination were subjected to short duration heat preconditioning at 35 °C for 3 h every third day until five weeks after germination. Heat preconditioned and nonpreconditioned plants were then transferred to two high temperature conditions (challenging temperatures) of 30/23 °C and 35/28 °C. A group of plants were grown at 25/18 °C throughout the experiment as control plants. These control temperatures were chosen because 18-25 °C is optimum range for greenhouse production of salvia (Nau, 1991). Temperatures of 28 to 35 °C commonly occur in transitional and warm climatic regions during mid-summer. Previous research indicated that temperatures of 30 to 35 °C deleteriously affected plant growth and thus were considered challenging temperatures. The media was kept moist at all times during the experiment.

Plant Growth and Development

The following plant growth parameters were measured at the end of each week. Plant heights were measured from the soil line to the apical meristem. Stem thickness and internode length were measured at the third internode from the apex. Stem thickness were determined using a digital calipers (VWR Scientific Instruments, Suwanee, GA) and averaging two measurements that were taken one perpendicular to the other. Number of days to flower was recorded when the first pair of flowers on the raceme inflorescence opened.

Net Photosynthesis, Stomatal Conductance and Transpiration.

CIRAS-1 portable photosynthesis system (PP systems, Amesbury, USA) was used to measure net photosynthesis, stomatal conductance, and transpiration. Measurements were recorded on recently matured leaves of eight week old plants; two measurements were recorded per plant.

Stomata Size, Stomatal Number, Leaf Thickness

Two recently matured leaf samples from each plant at eight week stage after germination were collected during the middle of the day and immediately taken to the Socolofsky Microscopy Center at LSU for processing to obtain SEM (scanning electron micrograph) images. Leaves were prepared for scanning electron microscopy(SEM).They were fixed in FAA (Ethanol, Glacial acetic acid, Formaldehyde), dehydrated in an ethanol series, and dried in carbon dioxide using Denton DCP-1 critical point drying apparatus. Then leaves were mounted on stubs, coated with 25 nm gold palladium using a Hummer II Sputter Coater, and examined on a Cambridge S-260 scanning electron microscope. Images of cross section, adaxial and abaxial surfaces were magnified to fixed resolution (300X for stomata, 200X for leaf cross section) in order to analyze the data. Standard electron micrograph images were used to measure the size of stomata, stomatal frequency and leaf thickness using Scion Image software (Scion Corporation www.scioncorp.com).

Plant Tissue Preparation, SDS PAGE, and Western Blotting

Leaf samples collected and stored after recording CIRAS-1 data were used for protein analysis. Frozen tissue samples from both heat stressed and non heat stressed plants were ground to fine powder using liquid N_2 and then extracted using the protocol from Downs et al., (1998) in buffer containing 100 mmol/L Tris-HCl (pH 8.0), 1%

sodium dodecyl sulfate (SDS; w/v), 1% dithiothreitol (DTT; w/v), 1 mmol/L phenyl methyl sulfonylfluoride (PMSF), 5 μmol/L leupeptin, 5 mmol/L ε-amino caproic acid, 1% ascorbate (w/v), and 3 mmol/L Na2EDTA (Sigma Chemical Co., St Louis, Missouri, USA). Three percent (w/v) polyvinylpyrrolidone (PVP) and/or 30 mmol/L sodium tetraborate was used to remove phenolics. Samples were boiled for 3 min and then centrifuged at 14000 g for 6 min. The supernatant was collected and stored at -80 °C.

Protein content from leaf extract was determined according to Bradford assay (Bradford, 1976), BSA was used as standard. The soluble proteins were fractionated on 15 % one dimensional SDS-PAGE gels as described previously by Laemmli (1970) and electro transferred to a 0.45μ nitrocellulose membrane (Towbin et al., 1979). After protein transfer, the nitrocellulose membranes were blocked in 1% (w/v) BSA and incubated with the primary and secondary antibodies. Molecular mass standards were included on all gels (Precision Plus Protein standards, BioRad, Hercules, CA). The relative amounts of protein-antibody complexes were estimated using a desktop scanner (Scanjet 3300C, Hewlett Packard, Palo Alto, CA) and ImageJ imaging software (ver1.33) (http://rsbweb.nih.gov/ij/)

Marketable Quality

Overall marketable quality was visually assessed weekly after heat stress treatments were initiated based on leaf color, terminal bud necrosis and flowering on 1 to 10 scale as described earlier, 1 being worst (necrotic leaves, poor flowering) and 10 being the best (healthy plants, green and well developed inflorescence). All the plants were destructively harvested to estimate the total leaf area per plant using Li-3100C leaf area meter (LI-COR environmental, Lincoln, NE), shoot and root dry weights at the termination of the experiment.

Experimental Design and Statistical Analysis

There were six experimental units per temperature treatment following a completely randomized design within the growth chambers. ProcMix and LSD adjusted by Tukey's method were performed to evaluate the significance of differences between parameters measured from different treatments at P=0.05. Simple correlation coefficients were obtained to analyze the association between marketable quality and growth parameters.

RESULTS

Plant Growth and Development

The influence of short duration heat preconditioning temperature on growth and development was evident from the morphological characteristics of both the salvia cultivars studied. Heat preconditioned plants when exposed to challenging temperatures showed no signs of wilting attributed to heat stress. The leaves of nonpreconditioned plants wilted immediately after exposure to challenging temperatures (personal observation). Heat preconditioned plants showed a remarkable change in overall appearance of whole plant by reduced internode length, leaf orientation and stem thickness compared to control (Fig 5.1). Percent reduction of plant height in nonpreconditioned Sizzler was significantly greater than Vista grown at all temperature treatments and preconditioned Sizzler plants (Fig.5.2A). Both the cultivars grown at challenging temperatures with and without heat preconditioning resulted in increased stem thickness (data not shown) and Vista had a greater increase in stem thickness at 35/28 °C challenging temperature when compared to Sizzler at both challenging temperature (Fig.5.2B). Heat preconditioning resulted in greater increase in stem

thickness at 30 °C for Sizzler approximately 44 % and at both the challenging temperature 55 % and 63 % for Vista.

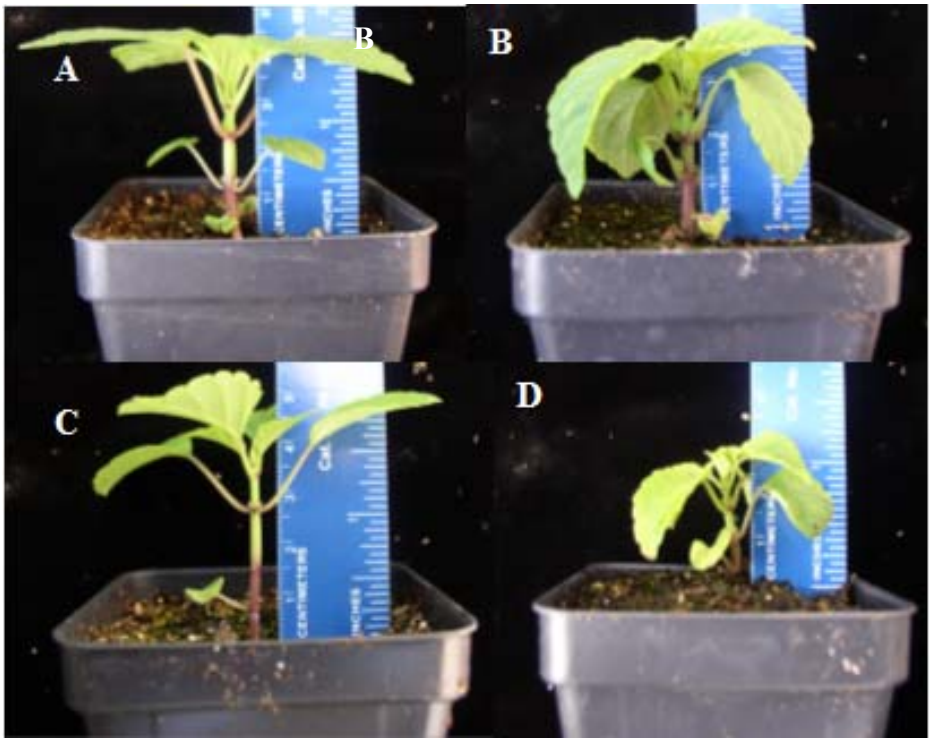

Figure 5.1 Effect of heat preconditioning (35 °C for 3 h every third day for 5 weeks) on overall seedling structure of *S.splendens* A) Vista control B) Vista preconditioned C) Sizzler control and D) Sizzler preconditioned.

Plant dry matter accumulation varied by cultivar and among treatments. Results showed that Vista are more efficient in dry matter accumulation when compared to Sizzler (Fig. 5.3A&B). Heat preconditioning helped Sizzler plants to maintain greater shoot growth and greater root growth at 30/23 and 35/28 °C challenging temperatures. Vista showed no significant changes in shoot dry weights over the control however root growth was greater at 35/28 C and preconditioned (Fig 5.3B).

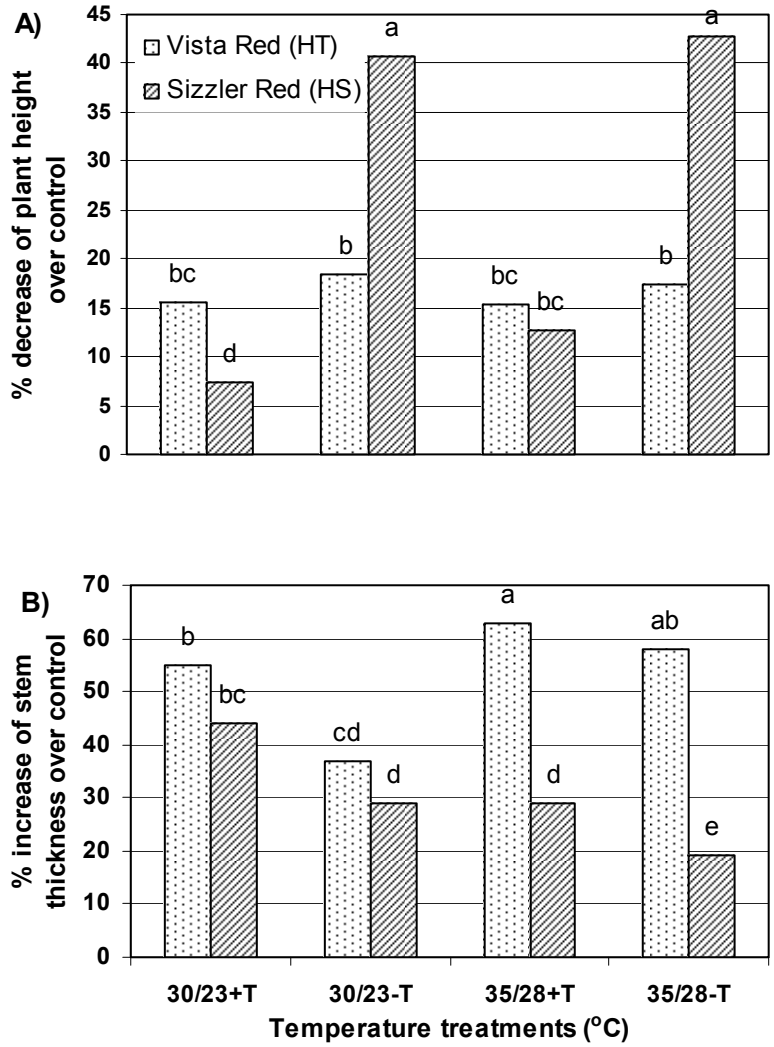

Figure 5.2. Effect of heat preconditioning (35 °C for 3h every third day for 5weeks) on per cent change in A) plant height and B) stem thickness in *Salvia splendens* cultivars 'Vista Red' and 'Sizzler Red' when exposed to two challenging temperatures 30/23 °C and 35/28 °C. (+T heat precondition; -T no precondition. HT heat tolerant; HS heat sensitive).Columns with different letters are significantly different at $P<0.05$ (Tukey's test).

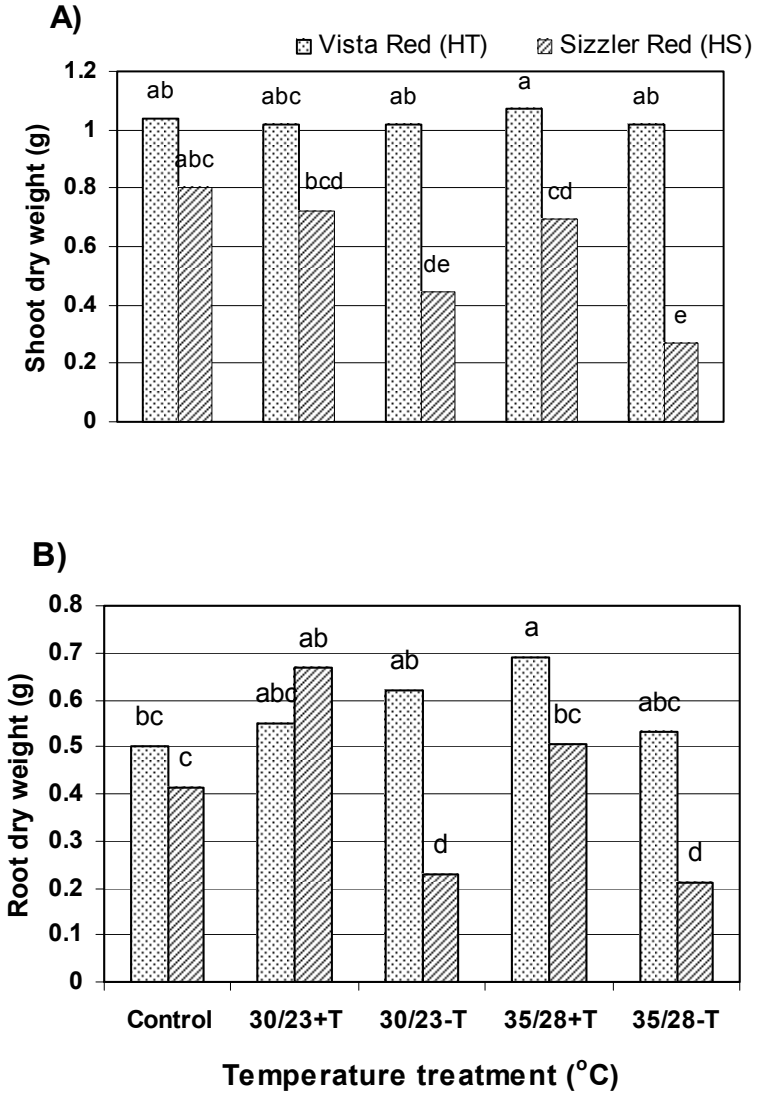

Figure 5.3. Effect of heat preconditioning (35 °C for 3h every third day for 5weeks) on A) shoot and B) root dry weights in *Salvia splendens* cultivars 'Vista Red' and 'Sizzler Red' when exposed to two challenging temperatures 30/23 °C and 35/28 °C. (+T heat precondition; -T no precondition. HT heat tolerant; HS heat sensitive). Columns with different letters are significantly different at $P<0.05$ (Tukey's test).

Stomata Size, Stomatal Number, Leaf Thickness

Comparison of the two cultivars showed significant differences in leaf anatomical characteristics under both control and challenging temperature treatments. Under challenging temperatures Vista showed enhancement of already existing heat tolerance traits than Sizzler. Vista had higher stomatal frequency on adaxial surface, and larger stomatal apparatus than Sizzler (Table 5.1). Both the cultivars in control condition had no significant difference in stomata number and size. Heat preconditioned plants of Vista at 35/28 °C challenging temperature had a greater stomata size than the control, non preconditioned Vista at 30/23 °C and Sizzler at all temperature treatments. Preconditioned Sizzler and subsequent growth at 30/23 °C had less number of stomata per unit area compared to preconditioned and grown at 35/28 °C and Vista at 35/28 °C temperature, with and without preconditining.

Vista with and without heat precondition treatment and subsequent growth at 35/28 °C and preconditioned plants at 30/23 °C challenging temperatures resulted in significant increment of leaf thickness over control and Sizzler at all treatments (Fig. 5.4). Increase in leaf thickness was greater under highest challenging temperature when compared to 30/23 °C challenging temperature for Vista. Sizzler showed an increase in leaf thickness at 30/23 °C challenging temperature over control, thickest leaves are observed only under 30/23 °C.

Net Photosynthesis (Pn), Stomatal conductance(Gs) and Transpiration(T).

Comparison of Vista with or without heat preconditioning at each challenging temperature showed no difference in T, Gs and Pn, but these parameters are greater at 35/28 °C when compared rest of the treatments (Fig.5.5A, B &C). Also as challenging temperature increased T and Pn of Vista. Stomatal conductance of Vista measured

Table 5.1. Stomata number and size on adaxial surface of *Salvia splendens* leaves

Cultivar Treatment (°C)	Stomata number no/mm^2	Stomata size µm^2
Vista Red		
Control	220.40 abc[y]	379.20 bcd
30/23(+T)[z]	222.22 abc	449.94 ab
30/23(-T)	218.58 abc	370.01 cd
35/28(+T)	261.51 a	485.62 a
35/28(-T)	251.11 a	436.74 abc
Sizzler Red		
Control	181.48 bc	340.86 d
30/23(+T)	162.96 c	348.30 d
30/23(-T)	170.37 bc	348.92 d
35/28+T)	233.33 ab	318.18 d
35/28(-T)	212.96 abc	324.29 d

[y]Values with different letters within columns are significantly different at P<0.05.
[z](+T) and (–T) are heat precondition and nonprecondition treatment respectively.

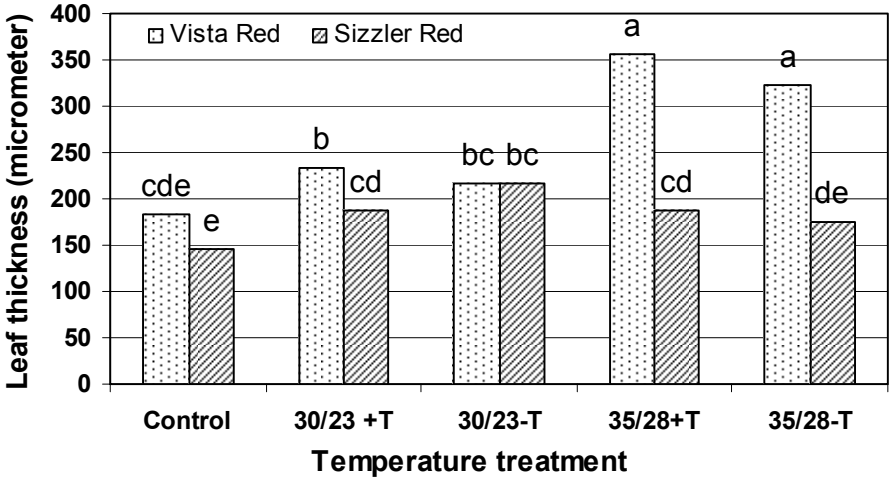

Figure 5.4. Effect of heat preconditioning (35 °C for 3h every third day for 5weeks) on leaf thickness in *Salvia splendens* cultivars 'Vista Red' and 'Sizzler Red' when exposed to two challenging temperatures 30/23 °C and 35/28 °C. (+T heat precondition; -T no precondition. HT heat tolerant; HS heat sensitive). Columns with different letters are significantly different at P< 0.05 (Tukey's test).

at 35/28 °C challenging temperatures was significantly greater than control plants. For Sizzler both preconditioned and non preconditioned plants at 35/28 °C had higher transpiration rate compared to control and preconditioned plants at 30/23 °C treatment. Net photosyntheis was greater at 35/28 °C in preconditioned Sizzler compared to control, preconditioned plants at 30/23 °C and non preconditioned plants at 35/28 °C. Comparing Vista and Sizzler, preconditioned Vista had maximum T, Gs and Pn at 35/28 °C.

Western Blotting

All the plants under control and two challenging temperatures with and without preconditioning resulted in synthesis of sHSP of approximately 27kD for Vista (Fig. 5.6 A). The intensity of bands however, increased under challenging temperatures. Sizzler synthesized these proteins in leaf samples of non preconditioned 30/23 °C grown plants and at both non preconditioned and preconditioned plants under 35/28 °C (Fig 5.6B).

Marketable Quality

Non-preconditioned Sizzler plants showed severe marginal burning of leaves, apical bud damage and poor flowering than control and Vista plants (Fig 5.7). High temperature treatment declined marketable quality of both the cultivars. However Vista with or without heat preconditioning had better marketable quality at both challenging temperatures than non-preconditioned Sizzler (Table 5.2). Percent reduction in marketable quality of non preconditioned Sizzler at the two challenging temperatures was 25 % and 58.9 % compared to control. Preconditioning helped Sizzler compared to non preconditioned plants by reducing the percent reduction in marketable quality over control at both the challenging temperatures. Correlation coefficients for associations of marketable quality with plant height, stem thickness, shoot and root dry weights showed a negative and significant correlation with plant height (Table 5.3). Overall marketable

quality primarily depended upon shoot and root growth characteristics and stems thickness to a lesser extent.

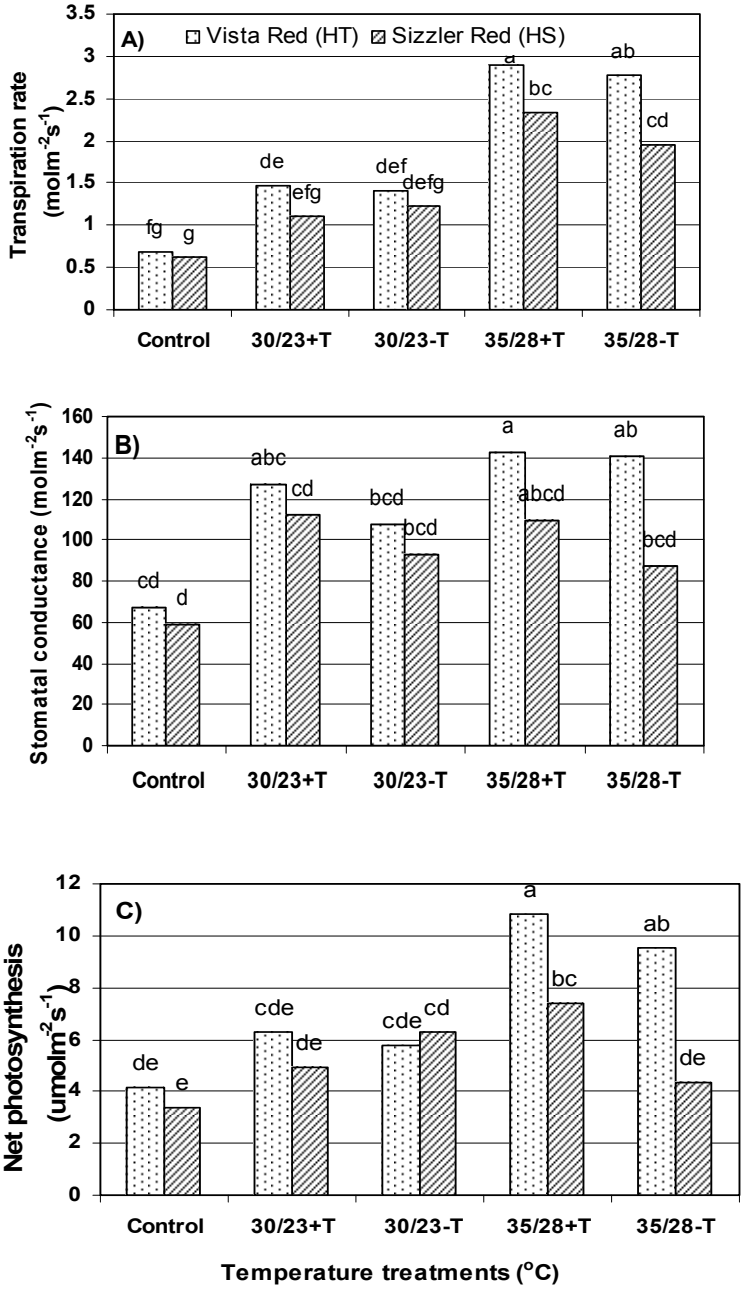

Figure 5.5. Effect of heat preconditioning (35 °C for 3h every third day for 5weeks) on A) transpiration B) stomatal conductance and C) net photosynthesis in *Salvia splendens* cultivars 'Vista Red' and 'Sizzler Red' when exposed to two challenging temperatures 30/23 °C and 35/28 °C. (+T heat precondition; -T no precondition. HT heat tolerant; HS heat sensitive). Columns with different letters are significantly different at $P<0.05$.

Table 5.2.Effect of heat preconditioning (35 °C for 3h every third day for 5weeks) on overall marketable quality of *Salvia splendens* cultivars 'Vista Red' and 'Sizzler Red' when exposed to two challenging temperatures 30/23 °C and 35/28 °C. Values in parenthesis are percent decrease over control. Values with different letters are significantly different at $P<0.05$. Quality score of 1= poor and 10=best.

Cultivar	Temperature treatments (°C)				
	Control	**30/23+T**	**30/23-T**	**35/28+T**	**35/28-T**
Vista Red (HT)	9.55 a	9.3 (-1.8) a	8.83(-7.0) a	8.5(-10.5) ab	8.30 (-12.2) abc
Sizzler Red (HS)	9.33 a	8.2 (-12.5) ab	7.00 (-25) cd	8.1 (-12.5)ab	3.83 (-58.9)d

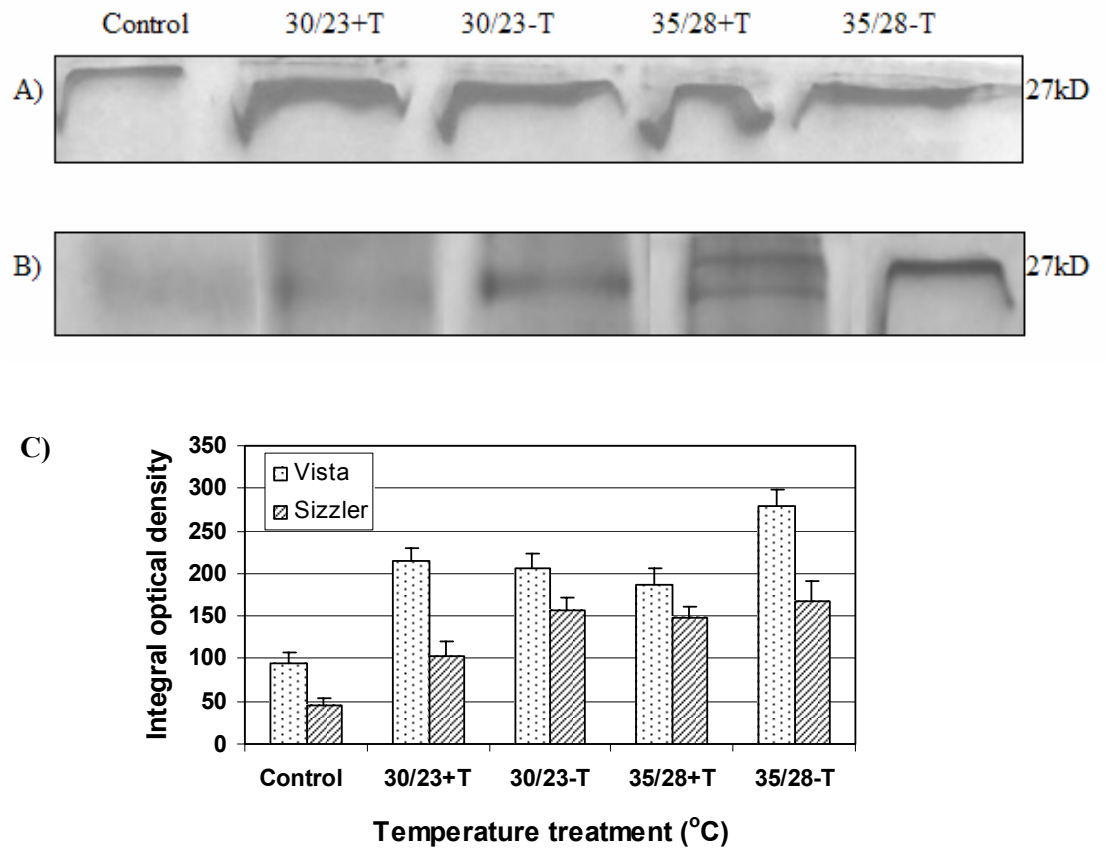

Figure 5.6.Effect of heat preconditioning (35 °C for 3h every third day for 5weeks) and exposure to two challenging temperatures 30/23 °C and 35/28 °C. (+T heat precondition; -T no precondition. HT heat tolerant; HS heat sensitive) on synthesis of sHSP27, in *Salvia splendens* A) Vista (heat tolerant). B) Sizzler (heat sensitive) C) Integral optical density values measured using ImajeJ software.Error bars indicates means of six observations ±SE

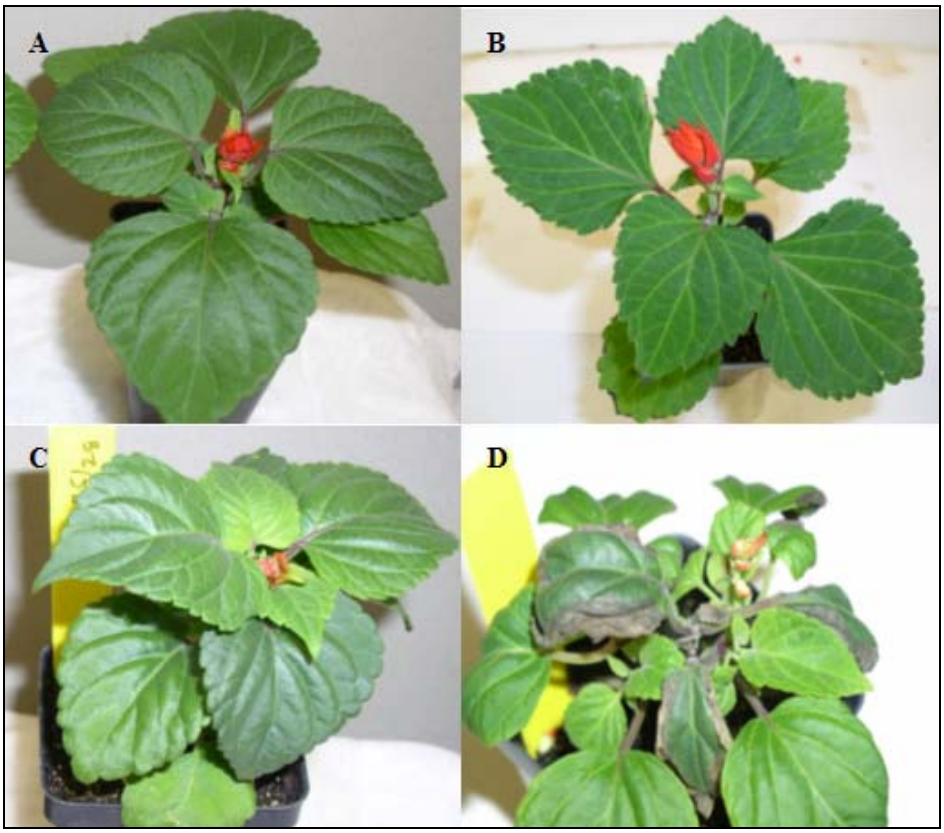

Figure 5.7. Effect of high challenging temperatures (35/28 °C) without heat preconditiong treatment (-T) on *S.splendens* cultivars A) Vista control B) Sizzler control C) Vista 35/28 °C -T and D) Sizzler 35/28 °C -T

Table 5.3. Correlation coefficients for association of marketable quality of salvia (*Salvia splendens*) with morphological traits plant height, stem thickness, shoot and root dry weights.

	Plant height	**Stem thickness**	**Shoot DW**	**Root DW**
Marketable Quality (P values)	− 0.581 (< 0.0001)	0.298 (0.0206)	0.722 (< 0.0001)	0.538 (< 0.0001)

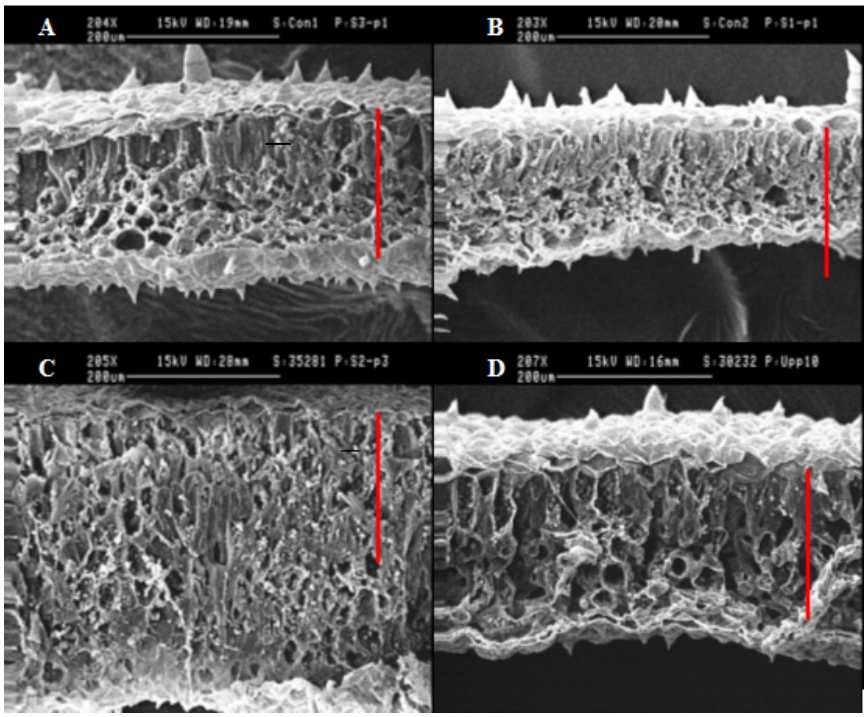

Figure 5.8. Leaf cross section SEM images at 200X magnification of *Salvia splendens.* Vista Red control B) Sizzler Red control C) Vista Red 35/28 °C +T D) Sizzler Red 35/28 °C +T. (+T Heat preconditioning at 35 °C for 3h every third day for 5 weeks). Vertical Red line indicates 200µm scale.

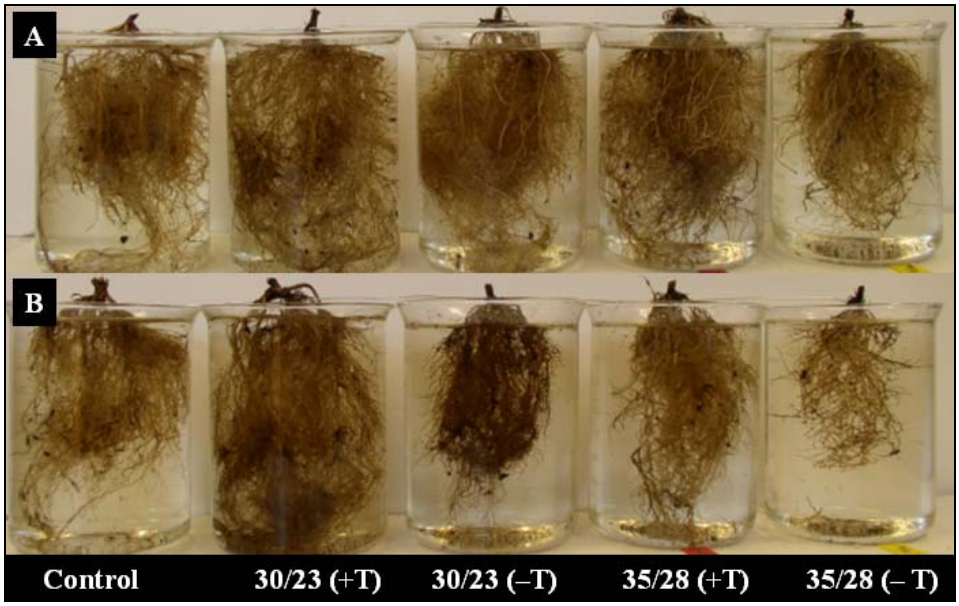

Figure 5.9. Roots suspended in 500 ml beakers showing, effect of heat preconditioning (35 °C for 3h every third day for 5 weeks) on root growth, in Salvia splendens A) 'Vista Red' (heat tolerant) and 'Sizzler Red (heat sensitive) when exposed to two challenging temperatures 30/23 °C and 35/28 °C. (+T heat precondition; -T no precondition).

DISCUSSION

In this study short duration heat preconditioning followed by growing salvia in challenging temperature revealed cultivar differences in acquired thermotolerance. Acquired thermoteolerance is defined as a phenomenon where, plants, when exposed to sublethal stress (induction stress), develop the ability to withstand severe temperatures (Senthil et al., 2003). In salvia this phenomenon of acquired thermotolerance was observed in all morphological, physiological and anatomical traits studied.

The two cultivars utilized in this study are different in their morphological growth patterns under control conditions as stated earlier (Personal correspondence PanAmerican Seeds Inc). These differences were enhanced when grown under challenging temperatures after heat preconditioning treatment for both the cultivars. In general the Vista with more heat tolerant morphological traits such as shorter plants, thicker stems, thicker and darker green leaves better shoot and root growth, are more heat tolerant than Sizzler lacking these traits. Sizzler, however after preconditioning developed some of these traits and acclimated to challenging temperatures. Vista maintained and enhanced already existing morphological traits and tolerated challenging temperatures. Griffin et al. (2004) reported that 21 days acclimation of Redbud (*Cercis canadensis*) seedlings at 35/25 °C showed an increase in leaf thickness and better plant growth and tolerated subsequent drought stress conditions. Seedling survival and recovery growth increased after 2 h induction treatment at 42 °C prior to different challenging temperatures in seedlings of sunflower (Senthil et al., 2003).

Results with respect to leaf thickness noticed in the present study, showed that both the cultivars developed thicker leaves under high temperature conditions, Vista however, are thicker than Sizzler. Increase in leaf thickness irrespective of cultivar could

be attributed to increase in cuticular thickening, mesophyll layer, and spongy parenchyma in both the cultivars at challenging temperatures (Fig 5.8). Leaf orientation also changed in both the cultivars after heat preconditioning (Fig 5.1). Gratani and Ghia (2002) found that the adaptive strategy of strawberry tree (*Arbutus unedo*) under mediterranean climate may be due to the high leaf area and leaf lamina thickness increasing the capacity of light interception, and gas exchange respectively, the steeper leaf inclination a prevention mechanism against photoinhibition.

Dry matter accumulation differed significantly among cultivars and treatments, percent reduction in dry matter accumulation could be attributed to changes in CO_2 assimilation among treatments and cultivars. Percent reduction of dry matter accumulation was found to be significantly less for heat preconditioned plants compared to non preconditioned plants of Sizzler (data not shown). Moreover heat sensitive Sizzler comparatively had significantly less shoot and root dry weights compared to heat tolerant Vista (Fig. 5.3A&B). Similar conclusions were made in earlier studies where heat tolerance of creeping bent grass was related to shoot density, canopy biomass, leaf blade width, and root biomass; better performance of heat tolerant variety was primarily due to its morphological traits (Beard 1999; Xu and Huang 2001).

Greater heat tolerance of Vista could be attributed to certain morphological traits of this cultivar such as improved root growth that in turn increased transpirational water movement with in the plant system. Increased transpiration rate reduced the leaf tissue temperature, above 38 °C however, lead to closure of stomata and leaf temperature increased, affecting the Rubisco activity and reduced net photosynthesis in maize (*Zea mays*) (Crafts-Brandner and Salvucii 2002). As temperature treatment increased transpiration rate and stomatal conductance increased for both Vista and Sizzler (Fig.

5.5). Sizzler always maintained lesser transpiration at all the temperature treatment which could be one of the reasons for damage symptoms of newly emerging as well as fully developed leaves (Fig. 5.7).

Transpiration is cooling mechanism of plants; however leaf temperature of young and emerging leaves increases more rapidly under heat stress. Because the new growth cannot cool itself adequately, heat stress results in foliar damage during exceptionally hot weather in late spring or early summer (Cotten, 1996). Lesser heat stress damage such as leaf marginal necrosis, of Vista could be due to their thicker leaves, particularly the thicker mesophyll layer (Fig. 5.8C). Nikolopoulos (2002) showed that adaptive advantages of *Quercus coccifera* and *Laurus nobilisin* with heterobaric leaves in xerothermic environments may be due to significant increase in leaf thickness and a consequent increase of the photosynthetic capacity per unit area. Species that are adapted to sunny and dry environments usually have small, thick and hard leaves (Givnish, 1987; Turner, 1994a, 1994b). These traits permit the greatest carbon gain per unit transpirational loss (Givnish, 1987).

Preconditioning and subsequent challenging temperature of 35/28 °C however, increased the transpiration rate and net photosynthesis of Sizzler series compared to non preconditioned plants (Fig. 5.5A&C). Increased net photosynthetic efficiency of preconditioned plants could be due to maximal quantum yield of PSII photochemistry and net CO_2 assimilation rate increased by preconditioning, as found in three *Cedar* species seedlings (Ladjal et al., 2000). Another unique molecular characteristic of plants and other thermophillic bacteria growing under high temperature condition is greater expression of heat shock protein synthesis (Knight and Ackerly 2003, Roy and Nakamoto, 1998).

Synthesis of heat shock proteins coincided with heat tolerance for Vista at both the challenging temperatures irrespective of preconditioning. While the results from Sizzler plants showed no direct relation between sHSP synthesis and plant performance under high temperature, even though these plants resulted in increased synthesis of sHSP compared to control plants. Earlier studies however, reported that preconditioning, sHSP synthesis and heat tolerance as related effects. Precondtioned plants resulted in greater accumulation of sHSP18 under subsequent lethal high temperatures of 35/27 °C in wheat (*Triticum aestivum*) (Ozkan, 1995). They also reported that, preconditioning at 37 °C for 20h improved survival capacity of cultivars 'Tosun' and 'Karachia'. Study conducted in transgenic tomato (*Lycopersicum esculentum*) reported that suppression HSP synthesis expression resulted in poor survival capacity of plants even after heat preconditioning treatment. Whereas wild type plants and plants that overexpresed HSP synthesis survived under high temperature after preconditioning at 45 °C for 1h (Mishra et al., 2002).

Plant quality and survival capacity under high temperatures depend upon root and shoot growth (Jiang and Huang 2001). Various studies reported that deep and extensive root system contribute positively to the increased water uptake under water heat stress conditions that increased plant quality and appearance (Sheffer et.al., 1987; Jiang and Huang, 2001). Root growth improved with heat preconditioning for both the cultivars (Fig5.9), Vista had relatively better root growth compared to Sizzler. Extensive root system would facilitate increased transpirational cooling by improved water movement, affecting reduced leaf temperature and thus heat tolerance (Engelke, 1985; Kolb and Robberecht, 1996; Xu and Huang, 2000). Bonos and Murphy (1999) reported that there was a significant difference in root growth between heat tolerant and heat sensitive cultivars of Kentucky blue grass at different depths of soils.

Our results agree with those of Jiang and Huang (2001) who also reported that preconditioning improved root growth, canopy photosynthesis and turf quality. Increased transpiration rate and stomatal conductance in Vista at 35/28 °C treatment may be attributed to their extensive root growth and leaf structure. Similar results were obtained in seedlings of tree species where stomatal conductance and transpiration rates are related positively to survival of seedlings under high temperature (Kolb and Robberecht, 1996). Comparing the overall plant growth, heat tolerant Vista were larger plants with greater leaf area, thicker stems and leaves, and shorter internodes which made them look compact. Collectively these traits are believed to be responsible fore reduced tissue temperature thus overall cooling of plants when soil moisture and root growth are not limiting.

Our results, in accordance with others, strongly suggest that maintaining certain morphological, anatomical traits and physiological adjustments are important for heat tolerance. Cultivars with compact canopy structure, thicker shoot structures, deeper and extensive root system, greater stomatal frequency and thicker palisade tissue would help in bedding plants such as salvia survive high temperature stress. Harbaugh and Scott (2001) reported that semi dwarf cultivars of Lisianthus (*Eustoma grandiflorum*) 'Florida pink' and 'Floida Blue' released as heat tolerant cultivars are more suitable as bedding plants in landscapes than cultivars with longer internodes. In a study conducted on cow pea cultivars Ismail et al. (2000) reported that heat-susceptible lines of cow pea were taller due to their longer internodes. Also prior exposure to sublethal temperature stress for a brief period might enhance these heat adaptable traits of plants that are not heat tolerant in recovery from heat shock (Levins, 1969; Vierling, 1991; O'Connell, 1994; Krebs and Loeschcke, 1994). Previous studies were concentrated either on drought

preconditioning or combination of drought and heat preconditioning, recently no work has been reported on heat preconditioning to improve heat tolerance in bedding plants. In the present study heat sensitive cultivar of salvia acquired thermotolerance after brief exposure to heat stress during the initial stages of seedling growth. Moreover this is the first report in bedding plant studies showing whole plant responses in terms of morphological, physiological and anatomical characteristics associated with induced or already existing thermotolerance.

In conclusion heat preconditioning for a brief period during the initial stages of plant growth increased the quality and survival capacity of plants in subsequent continuous heat stress of non heat tolerant Sizzler. Already existing heat tolerant traits of Vista were enhanced with preconditioning. The higher tolerance levels with and without heat preconditioning for Vista suggests that cultivar with broader leaves, higher stomatal frequency and thicker leaves had higher light interception, gas exchange, transpirational cooling and CO_2 fixation thus attained higher biomass accumulation to compensate the heat stress loss. These plants attained sooner canopy cover and were able to maintain healthier leaves and supply photoassimilates to reproductive development. These morphological, physiological and anatomical characteristics could be used to select for heat tolerant cultivars of Salvia and many other ornamental bedding plants.

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CHAPTER 6. HEAT TOLERANCE IN SALVIA (*SALVIA SPLENDENS)* CULTIVARS AS MEASURED BY ELECTROLYTE LEAKAGE TECHNIQUE

INTRODUCTION

Scarlet sage (*Salvia splendens*) is annual herbaceous bedding plants widely used in landscaping and home gardens. One of the predisposing factors leading to death of the garden salvia is high temperature stress. Although some cultivars of salvia can be grown in hot areas such as late spring and early summers of southern Gulf states, high temperatures of 30 to 35 °C have made it an erratic crop in production units and landscapes. The only available evaluation procedures for landscape plants include regional field trials (Albrecht and Pair, 1994; Bailey, 1998; Pemberton and Robertson, 2001). In addition to taking many years for selecting superior cultivars, this method is largely inefficient or not quantitative because of unpredictable heat stress events and interactions with other environmental factors. Therefore techniques for rapid and easy identification of heat tolerant genotypes during breeding and selection programs are highly desirable.

Cell membrane thermostability (CMT), calculated by measuring electrical conductivity of effluents or electrolytes from leaf discs exposed to a range of temperatures is a sensitive and rapid method to evaluate heat tolerance in plants (Wu and Wallner, 1993). Effectiveness of this technique was documented by several studies for detecting genetic variability for heat tolerance among agronomic, fruit, vegetable, turfgrass and floricultural crops (Chen et al., 1982; Ingram and Buchanan, 1984; Lester, 1985; Martineau et al., 1979; Saadalla et al., 1990; Sullivan and Ross, 1979; Walner et al., 1982; Yeh and Lin, 2003). Because of the wide range of temperatures used this process to derive percent injury response curves while screening several cultivars at one

can be tedious. Therefore for practical application of this technique, CMT measurement at a single temperature exposure at around 50 °C was effectively studied in groundnut (*Arachis hypogea*) (Chauhan and Senboku, 1996), pepper (*Capsicum annuum*) (Anderson et al., 1990), English Ivy (*Hedera helix*) (Yeh and Hsu, 2004), and turfgrass species (*Poa pratensis*) (Marcum, 1998). Since it is easier to setup a single temperature bath than to set up many different baths at several temperatures for CMT measurement (Yeh and Hsu, 2004), the single bath technique has potential to be used as a more efficient screening tool for heat tolerance.

Therefore a study was conducted to investigate the heat tolerance of *S. splendens* 'Vista Red' and 'Sizzler Red', and a known heat tolerant species *S. coccinea* 'Lady in Red' using a single bath technique to determine CMT by electrolyte leakage. Leaf relative water content, gas exchange capacity and marketable quality of these cultivars under high temperature stress was also measured to correlate with CMT.

MATERIALS AND METHODS

Plant Material

Salvia seeds were germinated in growth chambers (EGC, Chagrin Hills, OH) set at 25/18 °C in 10 cm pots Jiffy Mix® (Jiffy Products, Batavia, IL). Seedlings were grown in growth chambers with an average light intensity of 500 μmol m^{-2} s^{-1} with a 14 h photoperiod and a relative humidity of 75 to 80 %. Plants in the growth chambers were fertigated every morning at approximately 0900 h with 100 mg/L 15N-2.2P-12.4K (15-5-15 Cal Mg, Scotts-Sierra, Marysville, OH).

Temperature Treatment

Three week old seedlings of all three salvia were randomly arranged in growth chambers set at 25/18 °C (control) and 35/28 °C (heat stress) treatment for approximately

next seven weeks until flowering. Leaf RWC and marketable quality of plants are assessed every week on six plants per treatment from each cultivar.

Electrolyte Leakage Measurement at Single Temperature.

At approximately six week stage, 42 plants (six replicate plants × seven intervals of time 0, 10, 20, 30, 40, 50, 60 min) are moved from control to 35/28 °C for 1 day heat acclimation for CMT measurements. Two leaf samples were collected from fully expanded recently matured leaves for each replicate at the end of 1 day heat acclimation period. CMT was determined by a technique developed by Sullivan and Ross (1979) with modifications. Using cork borer four10 mm diameter leaf discs per leaf sample were punched. Leaf discs were thoroughly rinsed three times with deionized water in test tubes to remove any electrolytes adhering to, as well as electrolytes released from injured portion of the tissue. After final rinsing, tubes were capped with aluminum foil and placed in a hot water bath set at 50 °C. This temperature was chosen based upon previous experiments conducted with Vista and Sizzler cultivars where maximum separation of CMT response curve between cultivars was observed (CHAPTER 4).

Six tubes (replicates) of each cultivar were removed from the water bath at 10 min intervals for 60 min. After tissue incubation, the test tubes were allowed to cool to room temperature and 25 ml deionized water was added to the tubes and incubated at 10 °C for 24 h to allow electrolyte diffusion from leaf tissue. Then tubes were brought to 25 °C under room temperature condition and electrical conductivity was measured using a conductivity meter (VWR Scientific Instruments, Suwanee, GA). Tubes were then placed in autoclave at 0.1Mpa for 12 min in order to completely heat kill the tissue for maximum electrolyte leakage. Following autoclave killing, tubes were cooled down to 25 °C and

conductivity was measured. Maximum electrolyte leakage was used to calculate the percent electrolyte leakage following each time of exposure using the following formula:

$$\%\ electrolyte\ leakage\ = \frac{Electrolyte\ leakage\ at\ given\ time\ of\ \exp osure}{Total\ electrolyte\ leakage} \times 100$$

A sigmoidal curve was plotted with percent electrolyte leakage against time of exposure.

Leaf Relative Water Content Measurement (RWC)

Each week for six weeks, plants of each type of salvia grown at 25/18 °C (control) and 35/28 °C (heat stress) treatments were randomly selected for RWC measurement as described by Barr and Weatherly (1962). Leaf samples were collected and 2 cm diameter leaf discs, four discs per replicate plant, were cut using a cork borer. Initial leaf sample weight was recorded (W), after which samples were hydrated to full turgidity in vials for 4 h at 10 °C. After 4 h the leaf samples were taken out and dried quickly by gently wiping with filter paper to remove any surface moisture. Then immediately weighed to obtain fully turgid weight (TW). Samples were then dried completely in oven at 80 °C for 24 h and final sample dry weight weighed was recorded (DW). Leaf relative water content was measured using the following formula:

RWC (%) = [(W-DW) / (TW-DW)] x 100.

Where W equals sample fresh weight TW equals sample turgid weight, and DW equals sample dry weight.

Gas Exchange Measurements

Leaf gas exchange measurements were recorded after six weeks of growth on the 25/18 °C (control) and 35/28 °C (heat stress) plants using a CIRAS-I portable photosynthesis system (PP Systems, Amesbury, MA). Readings were taken on two recently matured leaves. One reading per leaf from six replicate plants for each treatment was taken inside the growth chamber.

Marketable Quality of Plants

At weekly intervals during the six week period, plants were visually rated for their marketable quality on a 1 to 10 scale; 1 being worst, 10 being best. Marketable quality scores 9 and 10 = excellent plants with healthy green leaves and good inflorescence, 8 = green healthy foliage with moderate flowers, 7 = plants with poor inflorescence, 5 and 6 = necrotic leaves and poor flower set, 4 = terminal bud damage, 2 and 3 = severely burnt leaves, 1 = dead.

Experimental Design and Statistical Analysis

All the plants from the three cultivars were randomly arranged in each of two growth chambers following a completely randomized design with in each chamber with six replicate plants for each cultivar. Data was analysed with SAS (Statistical Analyses System) using ProcMix and difference in means tested by Tukey's studentized test.

RESULTS

Percent Electrolyte Leakage

As time of exposure of leaf tissue to 50 °C increased , percent electrolyte leakage increased in a sigmoidal fashion for all the three cultivars (Fig 6.1). 'Vista Red' and 'Lady in Red' however, showed less membrane damage when compared to 'Sizzler Red' from 10 to 30 minutes of exposure. Beyond this exposure time all three cultivars showed similar tissue damage.

Leaf Relative Water Content (RWC)

Leaf relative water content of 'Vista Red' and 'Lady in Red' under heat stress (35/28 °C) remained equal to that of control (Fig 6.2). Where as RWC started to decline starting at week 5 and remained below the control treatment.

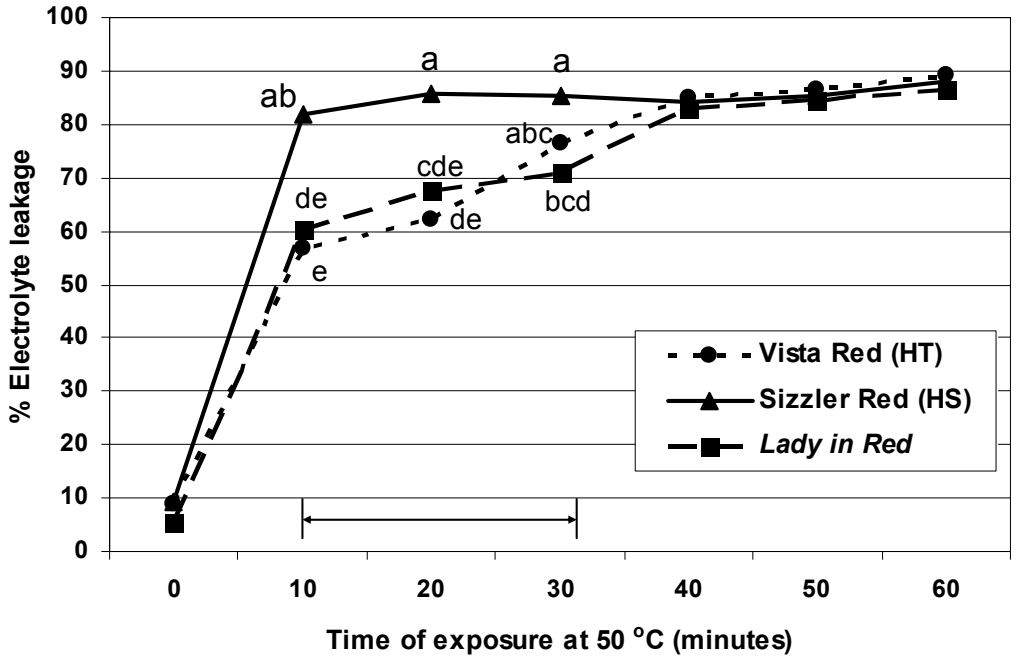


Figure 6.1. Effect of time of exposure of leaf tissue at 50 °C on percent electrolyte leakage in *Salvia splendens* 'Vista Red' and 'Sizzler Red' and *Salvia coccinea* 'Lady in Red'after a 24 h acclimatization at 35/28 °C of plants grown for six weeks at 25/18 °C. HT= heat tolerant, HS= heat sensitive. Means with different letters are significantly different at $P<0.05$ (Tukey's test). Sigmoidal fit r^2 for 'Vista Red'= 0.93; 'Sizzler Red'= 0.72; 'Lady in Red'= 0.87.

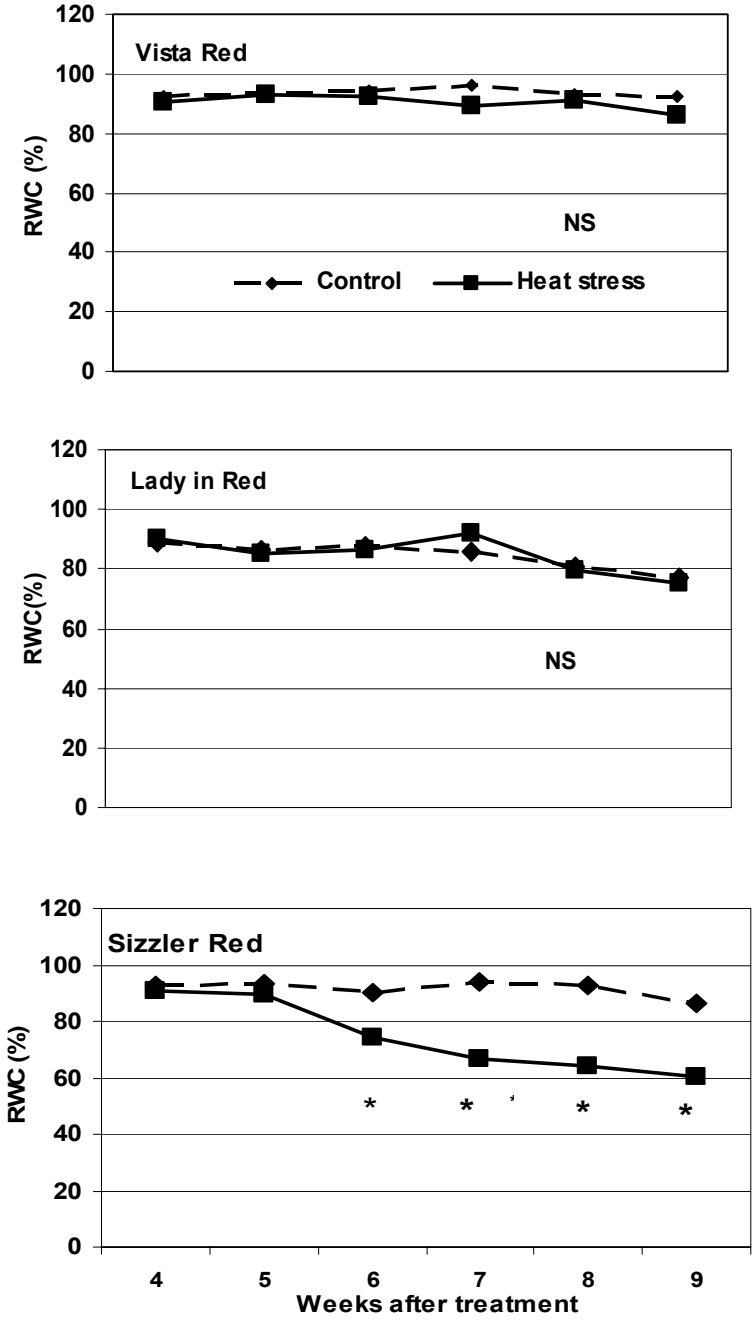


Figure 6.2. Leaf relative water content (RWC) at 25/28 °C (control) and 35/28 °C (heat stress) in *S.splendens* 'Vista Red', 'Sizzler Red' and *S.coccinea* 'Lady in Red' cultivars. Asterix indicates significant difference at P<0.05 (Tukey's test).NS=non significant.

Gas Exchange Measurements

Gas exchange measurements transpiration (T), stomatal conductance (Gs) and net photosynthesis (Pn) differed for all the three cultivars studied (Fig 6.4). Transpiration rate of plants grown under heat stress conditions increased for all the three cultivars compared to control. Stomatal conductance and net photosynthesis was greater for 'Vista Red' and 'Lady in Red' under heat stress condition compared to control. For 'Sizzler Red' stomatal conductance and net photosynthesis remained same as in control. Under heat stress condition 'Vista Red' and 'Lady in Red' maintained greater net photosynthetic rates compared to control and 'Sizzler Red' (Fig. 6.4C).

Marketable Quality

Heat stress treatment of 35/28 °C affected marketable quality compared to control for all the three cultivars. 'Sizzler Red' appeared to be most sensitive to high temperature, with significant decline in marketable quality after six weeks and decline below acceptable quality after seven weeks (Fig 6.5).

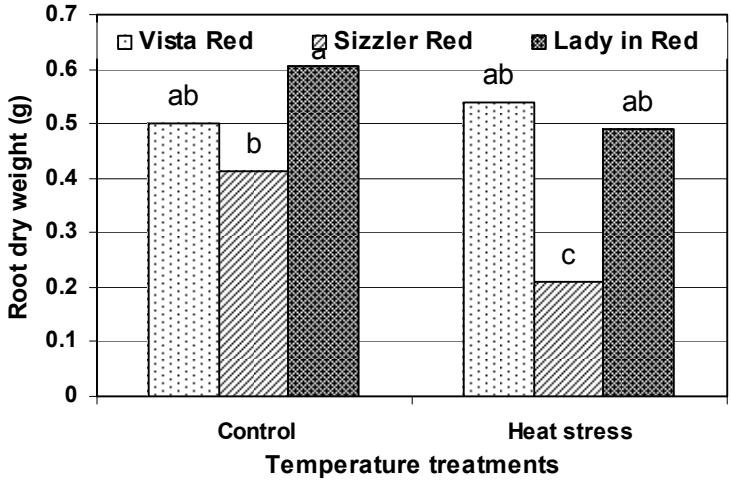
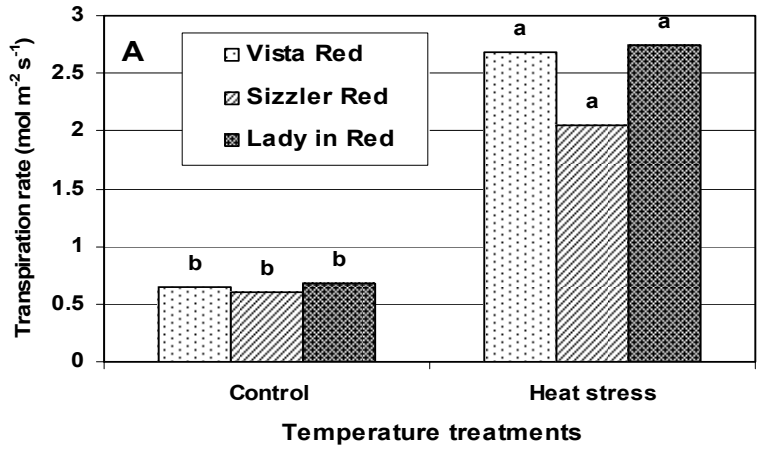
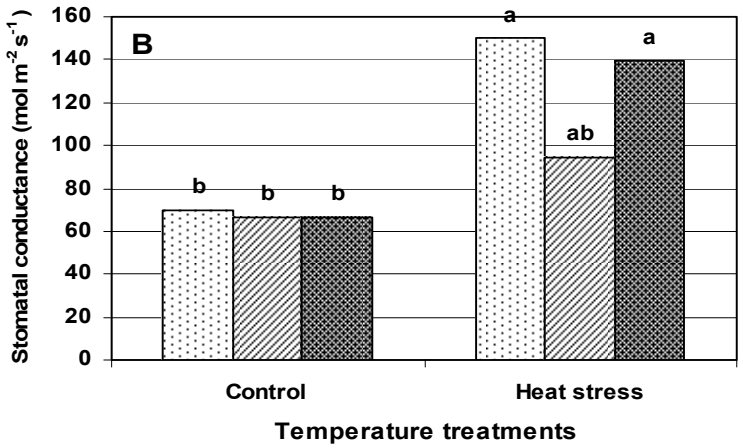


Figure 6.3. Root dry weight at 25/18 °C (control) and 35/28 °C (heat stress) in *Salvia splendens* cultivars Vista Red (heat tolerant) and Sizzler Red (heat sensitive) and *Salvia coccinea* 'Lady in Red'. Columns with different letters are significantly different at $P<0.05$ (Tukey's test).





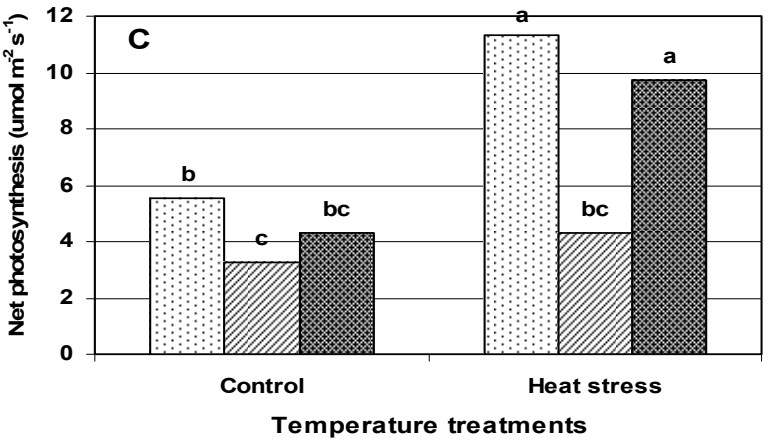


Figure 6.4. A) Transpiration B) stomatal conductance and C) net photosynthesis of six week old heat stressed *S.splendens* 'Vista Red', 'Sizzler Red' and *S.coccinea* 'Lady in Red' cultivars grown under control (25/18 °C) and Heat stress (35/28 °C) conditions. Columns with different letters are significantly different at $P<0.05$ (Tukey's test)

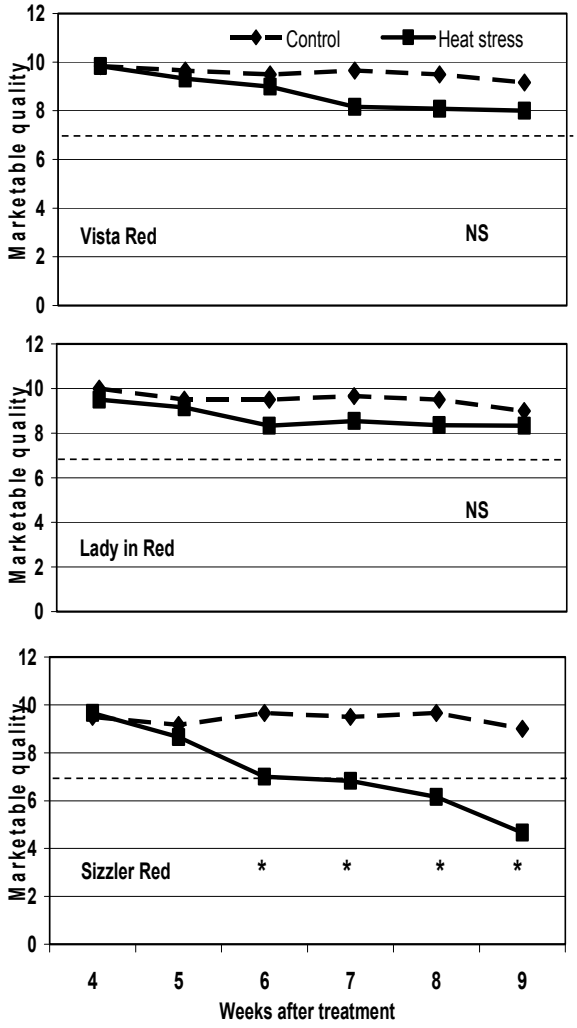


Figure 6.5. Marketable quality (10=best to 1= worst) as affected by 35/28 °C (Heat stress) in *Salvia splendens* cultivars 'Vista Red' (heat tolerant) and 'Sizzler Red' (heat sensitive) and *Salvia coccinea* 'Lady in Red'. Asterix represents significant difference at P<0.05 (Tukey's test). NS=non significant. (- - - -) indicates acceptable marketable quality. Qulaity score of 1= poor and 10= best.

DISCUSSION

From this study and based on previous experiments the CMT test or electrolyte leakage test, proved to be one of the most efficient methods of screening bedding plants for heat tolerance. Similar conclusions were obtained by earlier studies in soybeans (*Glycine max*) and english ivy (*Hedera helix*) (Martineau et al., 1979; Yeh and Hsu, 2004). The ability to tolerate high temperature stress can be measured physiologically and therefore appears to be dependent upon genotype (Ibrahim and Quick, 2001). Screening plants for heat tolerance under field condition is not quantitative and can be ineffective because of interaction with several environmental factors. Therefore a more rapid and accurate method of screening such as CMT is desirable.

Detection of heat tolerance is most accurate if plants are acclimated for one day at high temperature treatment prior to exposures of leaf discs to increasing temperatures to obtain CMT curves (Chen et al., 1982; Saadalla, 1990). Although the multiple temperature technique for testing CMT differences is inexpensive, it was more efficient and just as accurate to setup a single temperature water CMT test. This single temperature treatment of 50 °C was successfully used to detect the difference in heat tolerance of salvia cultivars (Fig. 6.1). There was a sigmoidal relationship between percent electrolyte leakage and time of exposure at 50 °C for all the three cultivars tested. Similar studies reported a sigmoidal response of electrolyte leakage to high temperature in soyabean (*Glycine max*) (Martineau et al., 1979), melons (*Cucumus melo* L), (Lester, 1985), citrus (*Citrus* species) (Ahrens and Ingram, 1988) and turfgrass species (Marcum 1998). Shifting of sigmoidal curves with an increase in exposure time was associated with genotypic response to high temperature. Genotypic difference in the heat tolerance

levels in these three salvia cultivars was also evident from leaf RWC and gas exchange measurements.

Although acclimation at high temperature for one day is a quantitative measurement of heat tolerance, the use of sublethal heat stress for longer periods is more common than direct lethal temperature exposure and sudden heat killing (Chen et al., 1982). Therefore it was important in this study to determine plant performance under such heat stress condition. The three salvia varieties were exposed to heat stress (35/28 °C) three weeks after germination until flowering. Prolonged duration of exposure to sublethal heat stress significantly affected marketable quality, RWC and gas exchange measurements.

Whole plant heat tolerance in terms of plant quality and RWC was greater for 'Vista Red' and 'Lady in Red' and became more pronounced as duration of exposure increased (Fig. 6.5 & 6.3). Decline in marketable quality under heat stress seems to be closely related to RWC and gas exchange results. Under heat stress condition transpiration rate, stomatal conductance and net photosynthesis increased significantly over control for 'Vista Red' and 'Lady in Red'. Rizhsky et al. (2002) reported that measurement of stomatal conductance under heat stress is accompanied by opening of stomata, to enable the cooling of leaves via an enhanced transpiration stream. Since heat tolerant 'Vista Red' and 'Lady in Red' had better root growth compared to 'Sizzler Red' (Fig. 6.3) transpirational water loss is compensated by root water uptake. Leaf net photosynthetic rates and Gs also depend on their leaf RWC (Lawlor, 1995; Cornic and Massacci, 1996; Lawlor, 2002a&b). Poor root growth of 'Sizzler Red' may have failed to supply enough moisture for transpiration water loss and hence net photosynthesis decreased. Under prolonged heat stress, a continued increase in transpiration results in

tremendous water loss from the plant leaf surface lowering RWC and increasing leaf temperature for sensitive species (Farkhutdinov et al., 2003). Although transpiration is a cooling effect, however when transpirational water loss exceeds moisture absorption, it results in increase in leaf tissue temperature and leaf burning symptoms affecting available photosynthetic surface (Halder and Burajee, 2003).This may results in overall reduced marketable quality of bedding plants.

In summary, heat stress affected marketable quality of all the three cultivars tested. Marketable quality of heat sensitive cultivar however, started to decline after three weeks of heat stress treatment and by the time of flowering they were below the level of acceptable quality (< 7). There was a close association between percent electrolyte leakage, leaf RWC, gas exchange and marketable quality and the degree of heat tolerance. This indicates that the single temperature CMT technique was appropriate for screening heat tolerant cultivars of salvia. Since single temperature CMT technique was accurate, rapid and less expensive than whole plant screening or CMT with a range of temperature exposure, this technique could be effectively used for screening other bedding plants on a large scale for heat tolerance.

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CHAPTER 7. HEAT STRESS RESPONSES AND SHORT DURATION HEAT PRECONDITIONING TO INDUCE HEAT TOLERANCE IN PANSY (*VIOLA X WITTROCKIANA*)

INTRODUCTION

Pansies or violas (*Viola x wittrockiana*) are one of the most commonly grown and commercially important ornamental crops. Among the specific bedding plants in the survey, pansy/viola flats contribute the second largest amount of $109 million next to impatiens (USDA Floriculture Crop Summary, 2004). Despite their importance little assessment of their growth and development response to environmental stress has been studied (Adams et al., 1997). High temperature stress is one of the primary environmental factors which influence the growth and development of many plants during the late spring and summer in most parts of the world (Koh, 2002). Extreme high temperature often leads to several closely related abiotic stresses such as water deficit and drought stress influencing many morphological, physiological and biochemical responses in plants. Heat injury in plants, as defined by Levit (1980), results from short exposure with in seconds to 30 min to extreme temperatures 45 to 65 °C. In supraoptimal temperatures, soil temperatures often reach injuriously high levels affecting root growth, strongly influencing shoot growth, leaf senescence and survival of whole plants. (Aldous and Kaufmann, 1979; Kuroyanagi and Paulsen, 1988; Paulsen, 1994; Udomprasert et al., 1995; Xu and Huang, 2004).

Merritt and Kohl (1991) reported a significant decrease in shoot dry weight of pansy 'Universal Violet' when grown at warmer night temperatures (27 day/18 °C night compared with 27 day/7 °C night). The rate of main stem leaf production in pansy was linearly related to temperature up to a maximum of 28.8 °C. (Adams et al., 1997).

Furthermore the flower size in pansy decreased with increase in temperature (Pearson et al., 1995). Guilioni (1997) reported that a short period of heat stress could cause a siginificant rate of floral bud abortion in pea (*Pisum sativum*), tomato (*Lycopersicum esculentum*) (Levy et al., 1978), snap bean (*Phaselous vulagris*) (Konsens et al., 1991), and cotton (*Gossypium* spp) (Reddy et al., 1992).

Physiological effects due to high temperature stress are increased respiration rate, reduced net photosynthesis, loss of cell water content due to increased transpiration and turgidity leading to closure of stomata (Cowan., 1977; Herve et al., 2002; Hubbard, 2001). Another signature physiological response of plants when exposed to sublethal heat stress is decreased synthesis of normal proteins accompanied by an accelerated increase of a group of proteins with a molecular mass of 15 to 42 kDa, designated as small heat shock proteins (sHSP) (Hsieh et al., 1992; Wehmeyer and Vierling, 2000; Lohmann et al., 2004). Plants are characterized by unusually abundant and diverse sHSP that may reflect their need to quickly adapt to ever changing environmental conditions such as temperature, light, and humidity (Sun et al., 2002). Small heat shock proteins act as molecular chaperones and protect biomolecules from detrimental effects of heat stress; increased levels of sHSP are positively correlated with heat tolerance (Clarke and Critchley, 1994; Heckathorn et al., 1996; Basha et al., 2004).

To overcome the effects of high temperature stress, plants also have evolved with remarkable ability to adapt/acclimate and survive in stressful environmental situations (Senthil et al., 2003). One of the primary plant adaptations to tolerate high temperature stress is commonly referred as acquired thermotolerance. Several studies with a number of different organisms, including plants, indicated that a brief exposure to sublethal high temperatures improves the ability of the organism to survive subsequent exposures to

potentially lethal temperatures (Levins, 1969; Vierling, 1991; O'Connell, 1994; Krebs and Loeschcke, 1994). Most of the plants have developed a wide range of adaptable features to high temperatures including morphological (smaller and narrower leaves, spines, reflective trichomes on upper leaf surface, deeper root system), physiological (high transpiration and stomatal conductance) (Thuiller, 2003; Xu and Huang, 2001) and anatomical traits (thick cuticle and thicker leaves) (Ristic, 1991). The capacity of plants to acclimate and survive under high temperature is a critical factor in heat tolerance. This adaptation and/or acclimatization to temperatures are considered as one of the primary determinants of geographical distribution of plants (Mahan et al., 1997).

Many bedding plants, however, remain difficult to grow prior to the season they are in most demand. For example, cool season bedding plants like pansy often undergo heat stress during late summer months and early fall resulting in reduced marketable quality and failure to survive in landscapes. Though intensive management practices can help alleviate heat stress in production units, they are often expensive and of limited effectiveness. Even though bedding plant production and landscaping have become the most important part of the floral industry ($2.53 billion) little work has been done to understand heat stress affects and mechanism of adaptation of these plants to high temperature stress. Therefore, understanding the morphological and physiological responses to heat stress, determination of the critical temperature where maximum damage occurs, and development of heat tolerant cultivars and/or practice proper cultural practices to reduce heat stress is highly warranted. The objectives of this research were 1) to study morphological (plant growth, dry matter accumulation, flowering) and physiological responses (transpiration rates, stomatal conductance, net photosynthesis, cell membrane stability, leaf soluble sugar content and synthesis of heat shock proteins)

of pansy cultivars to periodic short duration high temperature stress and 2) to investigate the effect of heat preconditioning and subsequent continuous heat stress affects on plant acclimation in terms of whole plant growth, development and, physiology as mentioned earlier.

MATERIALS AND METHODS

Plant Materials

Two experiments (Experiment 1 and 2) were conducted. Seeds of *Viola x wittrockiana* cultivars 'Crystal Bowl Purple' (CBP) and 'Majestic Giant Red' (MGR) were germinated in 10 cm pots filled with Jiffy Mix® (Jiffy Products, Batavia, IL) in environmental growth chambers (EGC, Chagrin Falls, OH). Heat tolerant CBP has short internodes, smaller sized leaves and flowers, late flowering and denser looking plant (highly branched). While heat sensitive MGR has longer internodes, is a robust looking plant with larger sized leaves and flowers, and few side shoots (personal correspondence American Takii Seeds). Growth chambers were programmed to maintain 25/18 °C day/night cycles with a 14 h photoperiod of 500 μmoles m^2 s^{-1} PPFD (photosynthetic photon flux density). Plants in the growth chambers were fertigated every morning at approximately 0900H with 100 mg/L 15N-2.2P-12.4K (15-5-15 Cal Mg, Scotts-Sierra, Marysville, OH).

Temperature Treatments

Experiment 1: Short Duration Heat Stress Responses

Four week old plants were subjected to short term high temperature treatments for 3 h on every third day in growth chambers. Temperature was gradually ramped up in 5 °C increment every 30 minutes from 25 °C to 30, 35, 40 and 45 °C. These high temperature treatments were maintained for 3 h and then ramped down similarly to 25

°C. These method of temperature treatments were based on similar studies using maize (*Zea mays*) (Crafts-Brandner and Salvucii, 2002) with some modifications. Temperature treatments were continued for approximately 14 weeks until completion of flowering to study the morphological and physiological responses of plants to short duration high temperature treatments.

Experiment 2: Heat Preconditioning and Subsequent Two Challenging Temperature Stress

Four week old seedlings after appearance of four to six true leaves were subjected to short duration heat preconditioning at 30 °C for 3 h every third day for a duration of four weeks. Heat preconditioned and non-preconditioned plants were then transferred to two high temperature treatments (challenging temperatures) of 30/23 °C and 35/28 °C. A group of plants was grown at 25/18 °C throughout the experiment as control plants. These test temperatures were choosen because 18 to 25 °C is optimum range for greenhouse production of pansy (Nau, 1991). Temperatures of 28 to 35 °C commonly occur in transitional and warm climatic regions during mid-summer. Previous research indicated that temperatures of 30 to 35 °C deleteriously affected growth of pansy and thus were considered challenging temperatures. Plants were fertigated every morning as described previously.

Plant Growth and Development

The same data was for both experiment 1 and experiment 2. Plant height was recorded from soil line to the apical meristem of the longest shoot and measured on individual finished plants. Internode length and number of branches were also recorded. All the plants were destructively harvested after flowering and total leaf area per plant, shoot and root dry weights were obtained.

Gas Exchange Measurements

Physiological parameters such as net photosynthesis (Pn), stomatal conductance (Gs), and transpiration (T) were measured on two recently matured leaves from eight week old plants using CIRAS-I portable photosynthesis system (PP systems, Amesbury, MA). Measurements were recorded 24 h after a heat stress treatment of 30, 35, 40 and 45 °C. One reading per leaf on all six replicate plants was recorded under artificial light source emitting equal light intensity as that in experimental growth chamber

Electrolyte Leakage

Electrical conductivity of electrolytes from leaf tissue was measured according to the method previously described (Raymond et al., 1986; Hallam and Tibbits, 1988) with some modifications. Eight week old plants grown at 25/18 °C were acclimatized for 24 h at 35/28 °C in the growth chamber. Five mm diameter leaf discs were punched from recently matured leaves on either side of the mid vein using a cork borer. Freshly collected leaf discs were washed twice with distilled deionized water in a test tube and after draining the water, test tubes were sealed with aluminum foil and incubated in thermostat controlled water baths set at 30, 35 40 45, 50, 55 or 60 °C for 1 h. After tissue incubation, the test tubes were cooled to room temperature and 25 ml deionized water were added to the tubes for incubation at 25 °C for 1 h, under agitation. Conductivity of water surrounding the leaf tissue was measured using a conductivity meter (VWR Scientific Instruments, Suwanee, GA). For measuring maximum membrane damage, test tubes with leaf discs were autoclaved for 15 min at 0.1 Mpa and conductivity of water was recorded. Electrical conductivity measurements were used to calculate lethal temperature (LT_{50}). LT_{50} is defined as the temperature at which there is 50% membrane damage determined by the conductivity of water surrounding the tissues treated at

different increasing temperatures (Ortiz and Cardemil, 2001). The following equation was used to calculate percent membrane damage:

$$\% \text{ of membrane damage} = \frac{Cx - Cc}{Cm - Cc} \times 100$$

Where *Cx* is the conductivity of water surrounding the leaf tissue incubated at different temperatures for 1 h, *Cc* is the conductivity of water of the sample at 25 °C, and *Cm* is the maximum conductivity after autoclaving the leaf tissue.

Total Water Soluble Sugar Content (WSS)

Leaves harvested after taking CIRAS-I data were immediately frozen in liquid nitrogen and stored at −40 °C until further analysis. Frozen leaf tissue was lyophilized, weighed, and ground to pass a 20 mesh screen. Water soluble sugars (WSS) were extracted and measured using the Miller and Langhans (1989) procedure three times with one milliliter (ml) with 12 methanol: 5 chloroform: 3 water (MCW) by volume. Fifty milligrams of finely ground leaf tissue was weighed into disposable Pasteur pipets fitted with glass wool plugs. One ml of MCW solution was added to each pipets, to rinse the sides of tube another 0.5ml of MCW solution was added and stirred with a glass rod and left to set for one hour. Then the pipets were drained with compressed nitrogen to drain the solution from the pipets to labeled 15 ml centrifuge tube. Mannitol (1.0 mg) was used as an internal standard and added at the beginning of the first MCW extraction. After extraction was complete, each pipet was rinsed with 1 ml MCW. After final drain of solution 3 ml of distilled deionized water was added to partition out the chloroform by centrifugation in a swinging bucket centrifuge for 20 min at 12000 g. After centrifugation the aqueus phase was removed and filtered through polyethylene columns containing 1 ml Amberlite IRA-68, acetate form, and 1 ml Dowex 50-W, hydrogen form (Sigma-

Aldrich, St.Louis, MO) resisns. Columns were washed twice with 0.5 ml Methanol : water (1:1).

The aqueus phase was then vaccum dried using an Evapotech^{TM} (Haake Buchler, Saddle Brooke, NJ) with a refrigerated condensation trap RT100 (Savant Ins. Farmingdale, NY) to concentrate the soluble sugars. To the concentrated sugars 2 ml of HPLC- grade water was added to resuspend and forced through a 0.45µm membrane filter using a 13mm plastic swinney filter holder (Pall, Gelman Lab, Ann Arbor, MI) to disposable vials. Filtered samples were injected into Waters HPLC system (Milford, MA) using HPLC-grade water as mobile phase at the rate of 1.0 ml per minute. Total water soluble sugars (WSS) were separated on a Shodex 1011 column (J M Sciences Inc. Grand Isaland, NY) maintained at 80 °C using a column heater. Total sugars were detected by refractive index and determination of specific sugars was based on the comparison of retention times to those of authentic D-sugars, Sucrose, Raffinose, glucose and fructose (Sigma # S-9378, # R-0250, #G-5250, and # F-0127, respectively).

Plant Tissue Preparation, SDS PAGE, and Western Blotting

Leaf samples collected and stored after recording CIRAS-1 data were used for protein analysis. Frozen tissue samples from both heat stressed and non heat stressed plants were ground to fine powder using liquid N_2 and then extracted using the protocol from Downs et al., (1998) in buffer containing 100 mmol/L Tris-HCl (pH 8.0), 1% sodium dodecyl sulfate (SDS; w/v), 1% dithiothreitol (DTT; w/v), 1 mmol/L phenyl methyl sulfonylfluoride (PMSF), 5 µmol/L leupeptin, 5 mmol/L ε-amino caproic acid, 1% ascorbate (w/v), and 3 mmol/L Na2EDTA (Sigma Chemical Co., St Louis, Missouri, USA). Three percent (w/v) polyvinylpyrrolidone (PVP) and/or 30 mmol/L sodium

tetraborate was used to remove phenolics. Samples were boiled for 3 min and then centrifuged at 14000 g for 6 min. The supernatant was collected and stored at -80 °C.

Protein content from leaf extract was determined according to Bradford assay (Bradford, 1976), BSA was used as standard. The soluble proteins were fractionated on 15 % one dimensional SDS-PAGE gels as described previously by Laemmli (1970) and electro transferred to a 0.45µ nitrocellulose membrane (Towbin et al., 1979). After protein transfer, the nitrocellulose membranes were blocked in 1% (w/v) BSA and incubated with the primary and secondary antibodies. Molecular mass standards were included on all gels (Precision Plus Protein standards, BioRad, Hercules, CA). The relative amounts of protein-antibody complexes were estimated using a desktop scanner (Scanjet 3300C, Hewlett Packard, Palo Alto, CA) and ImageJ imaging software (ver1.33) (http://rsbweb.nih.gov/ij/)

Marketable Quality

Overall marketable quality of plants from both the experiments was assessed based on 1 to 10 scale with 10 being the best and 1 being the worst. Marketable quality scores as 9 and 10 = excellent plants with healthy green leaves and flowers. 8 = green healthy foliage with moderate flowers, 7 = plants with poor inflorescence, 5 and 6 = necrotic leaves and poor flower set, 4 = terminal bud damage, 2 and 3 = severe marginal burning proceeding towards the center of leaf lamina or 1 = dead.

Experimental Design and Statistical Analysis

In both the experiments plants were arranged in a completely randomized design within each treatment chamber with six replicate plants per cultivar. Statistical analysis was performed using SAS (Statistical Analysis Software, version 9.0, Cary, NC). A

variance analysis, using ProcMix procedure of SAS was performed and significance of differences in mean was determined by Tukey's test.

RESULTS

Experiment 1

Plant Growth

High temperature treatment of 45 °C severely affected pansy seedlings of both the cultivars during the first few weeks of study and all seedlings in this treatment died. Periodic short duration high temperature treatments resulted in a decline in all the growth parameters studied. A short duration exposure of 40 °C reduced plant height significantly in both CBP and MGR (Fig. 7.1A). Between the two cultivars, CBP were shorter plants compared to MGR under control and high temperature treatment of 30 °C.

Number of branches/shoot was significantly greater for CBP when compared to MGR at control and high temperature treatments of 30 and 40 °C (Fig.7.1B). High temperature treatments reduced the number of CBP side branches. MGR produced fewer shoots at all the temperature treatments compared to CBP.Total leaf area per plant was unaffected by high temperature treatment for MGR (Fig 7.1C). At high temperature treatments of 35 and 40 °C total leaf area was reduced when compared to control for CBP. Total leaf area remained unchanged for MGR at all treatments. Shoot and root dry weights declined for both the cultivars as temperature treatment increased (Fig 7.2A &B). There was no difference between the two cultivars in shoot and root dry weights.

Transpiration (T), Stomatal Conductance (Gs) and Net Photosynthesis (Pn)

Transpiration rate increased at 30 °C for both CBP and MGR (Fig 7.3A). There-after CBP maintained constant transpiration rate at 35, 40, and 45 °C. MGR has greater transpiration rate at 35 and 40 °C and at the highest temperature treatment transpiration

rate declined below control. Between the two cultivars, CBP maintained a greater transpiration rate at 30 and 35 °C.

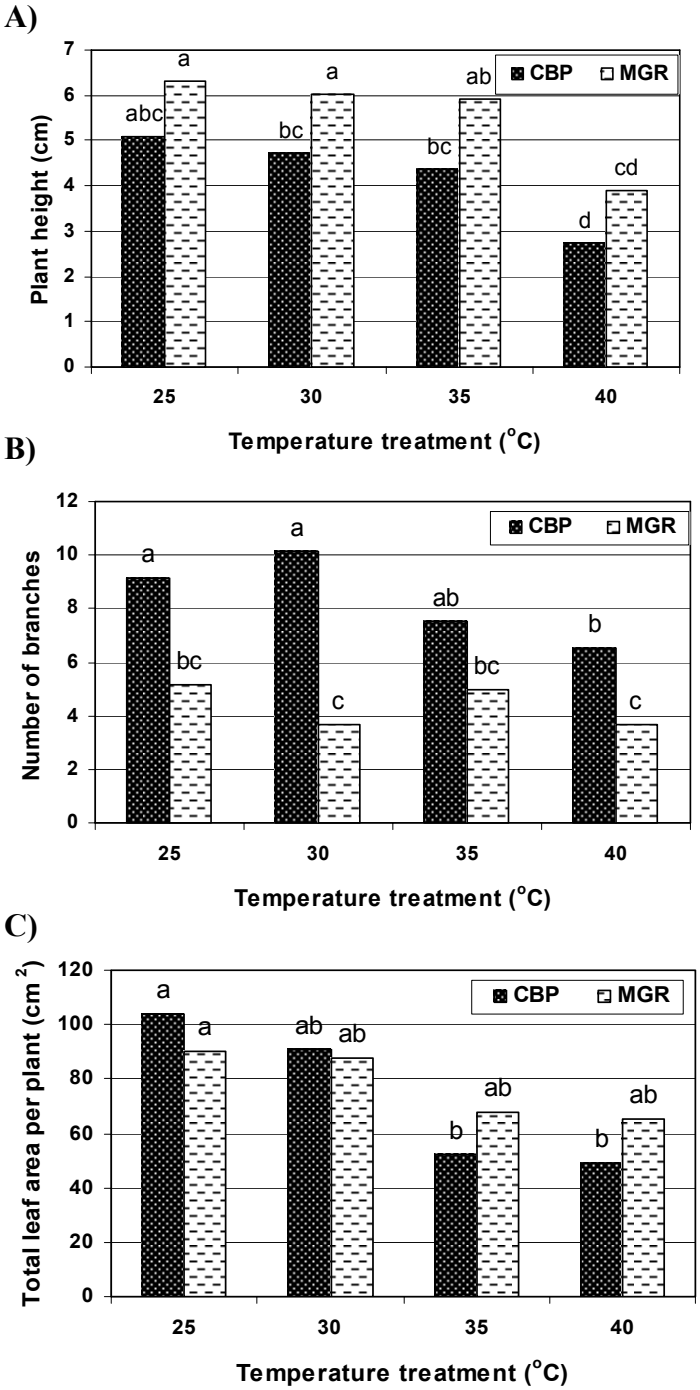

Figure 7.1. Effect of temperature treatment for 3h every third day on A) plant height B)number of branches C) Total leaf area per plant in *Viola x witrrockiana* Crystal Bowl Purple -CBP (heat tolerant) and Majestic Giant Red-MGR (heat sensitive). Means with different letters are significantly different at $P<0.05$ (Tukey's test).

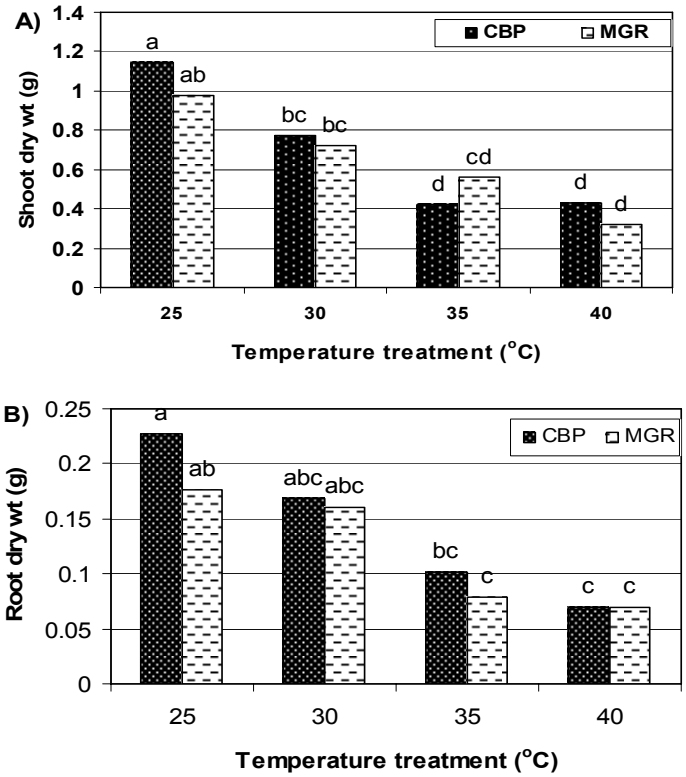

Figure 7.2. Effect of temperature treatment for 3h every third day on A) shoot dry weight B)root dry weight, in *Viola x witrrockiana* Crystal Bowl Purple -CBP (heat tolerant) and Majestic Giant Red-MGR (heat sensitive). Means with different letters are significantly different at $P<0.05$ (Tukey's test).

Stomatal conductance increased as temperature treatment increased above 30 °C for CBP (Fig.7.3B). CBP resulted in a greater stomatal conductance at 35 °C compared to MGR. The 45 °C treatment reduced stomatal conductance for both the cultivars below the control level.

Net photosynthesis increased with increase in temperature treatment from 25 °C to 30 °C for both the cultivars and decreased as temperature treatment increased above 30 °C (Fig 7.3C). Maximum net photosynthesis was recorded at 30 °C for both the cultivars however CBP had greater net photosynthesis compared to MGR. Above this temperature treatment both cultivars had similar rates of net photosynthesis but lower rates at 40 and 45 °C compared to control.

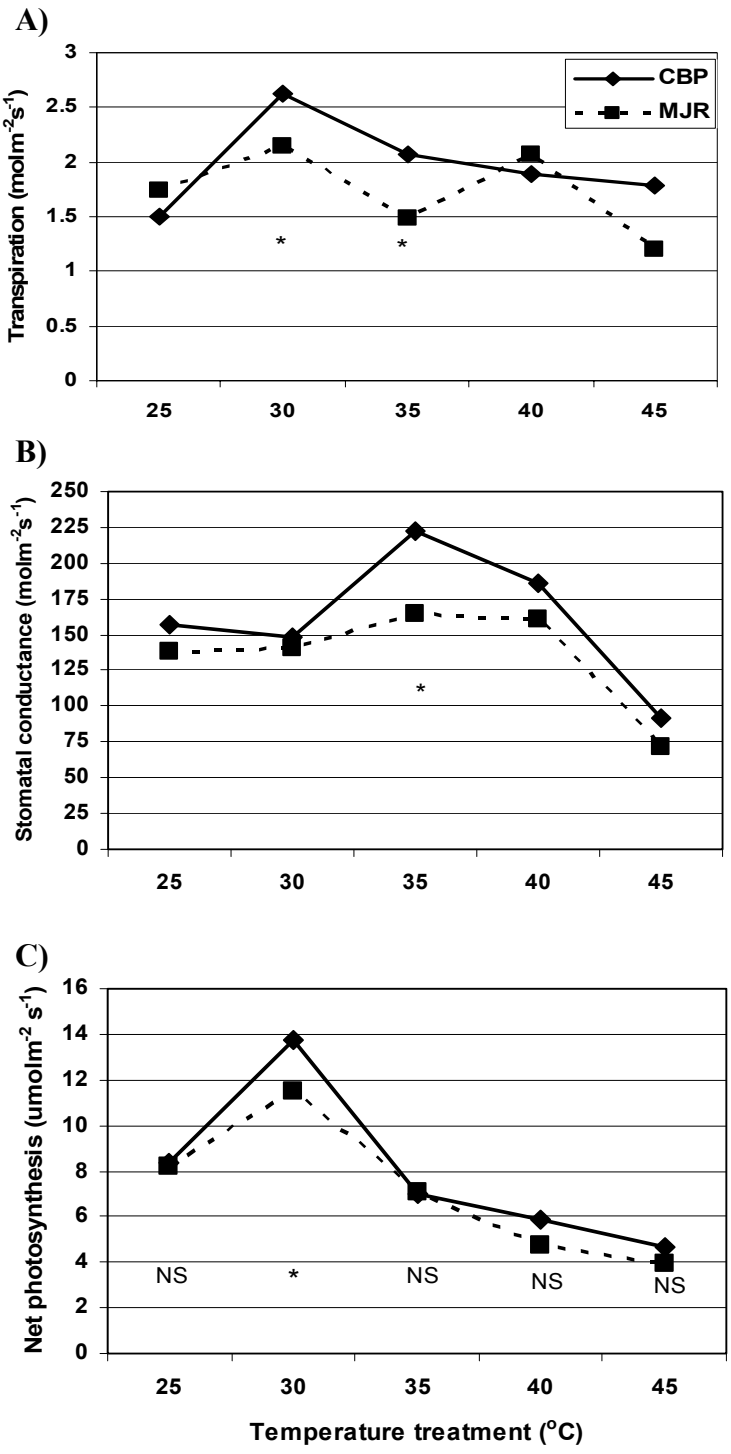

Figure 7.3. Effect of temperature treatment for 3h every third day on A) transpiration B)stomatal conductance C) net photosynthesis in *Viola x witrrockiana* Crystal Bowl Purple -CBP (heat tolerant) and Majestic Giant Red-MGR (heat sensitive). Means with different letters are significantly different at $P<0.05$ (Tukey's test)

Total Water Soluble Sugar Concentrations (WSS)

As temperature treatment increased, sucrose concentration increased up to 30 °C in comparison to the control. Sucrose concentration declined for both cultivars above 30 °C in comparison to the control (Fig.7.4A). CBP however had greater concentrations of sucrose at 30 °C compared to MGR and control plants.

Glucose concentration increased for CBP as temperature treatment increased from 25 to 30 °C and subsequently decreased as temperature treatment increased reaching below control level at 45 °C (Fig 7.4B). MGR showed an initial decrease in glucose concentration from 25 C to 30 °C and then increased as temperature treatment increased up to 35 °C, above 35 °C glucose concentration reached below control levels. At 30 °C CBP has higher concentrations of glucose compared to MGR.

Both the cultivars showed similar pattern of change in fructose concentrations as temperature treatment increased. At 35 °C both the cultivars showed greater concentrations of fructose compared to control and all the high temperature treatements (Fig 7.4C); however CBP had greater concentration compared to MGR.

Electrolyte Leakage

With increasing incubation temperature of water bath the percent membrane damage or the electrolyte leakage increased in a sigmoidal fashion (Fig 7.5). MGR showed a significant shift in membrane damage response curve starting at 45 and 50 °C, above this temperature treatment both the cultivars responded similarly to high temperatures. The LT_{50} temperatures indicated with arrows are the lethal temperatures for 50 % membrane damage of leaf tissue, a difference of 2.5 °C between two cultivars was observed.

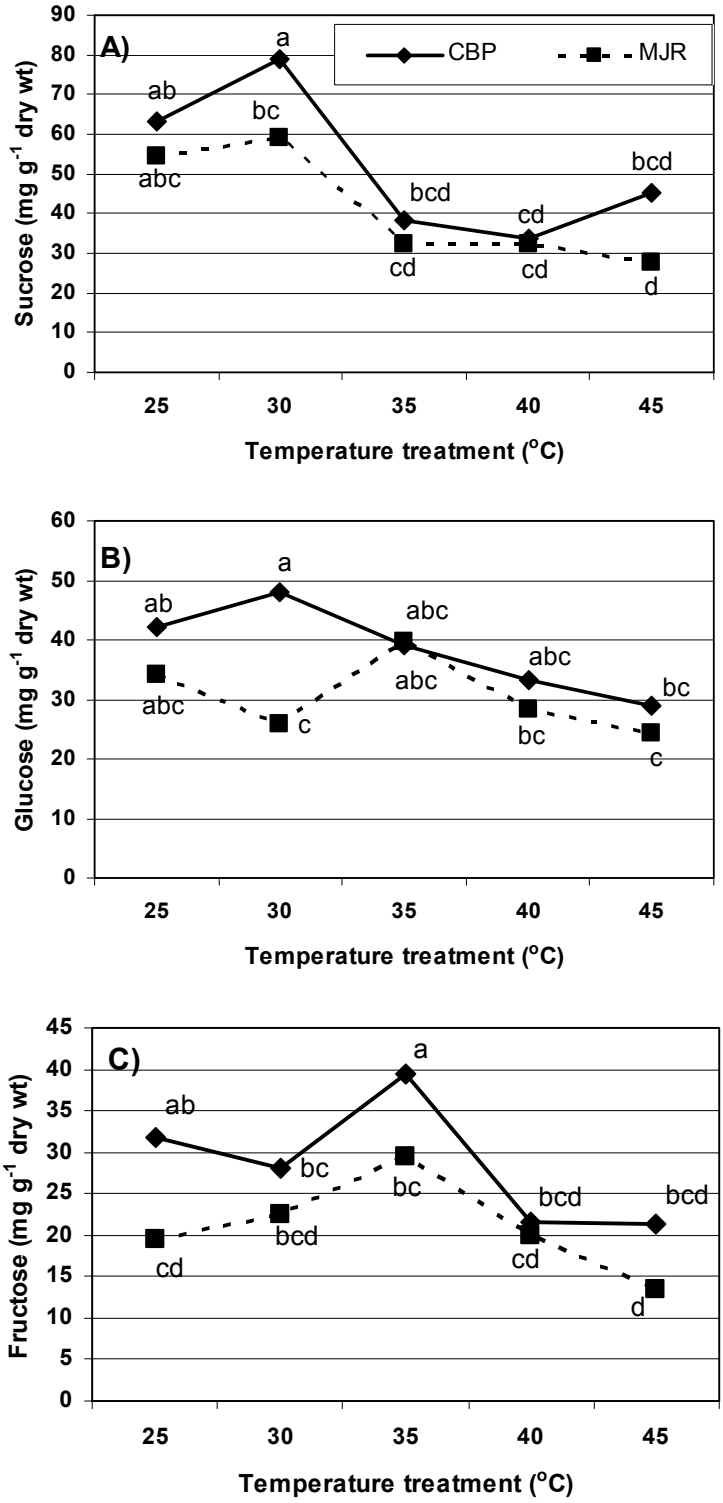

Figure 7.4. Effect of temperature treatment for 3h every third day on A) sucrose B)glucose C) fructose in *Viola x witrrockiana* Crystal Bowl Purple -CBP (heat tolerant) and Majestic Giant Red-MGR (heat sensitive). Means with different letters are significantly different at $P<0.05$ (Tukey's test).

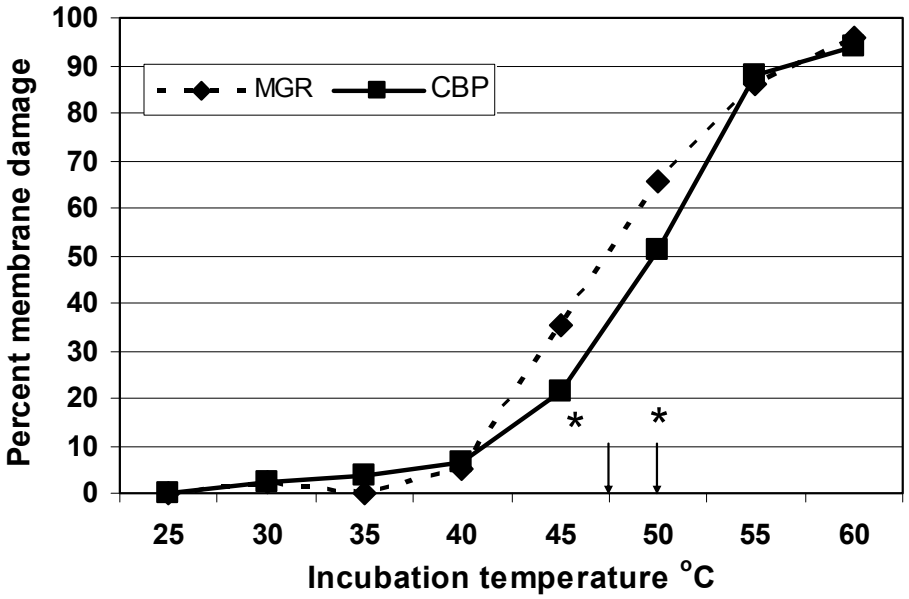

Figure 7.5 : Effect of short duration high temperature treatment percent membrane damage in leaf tissue of *Viola x wittrockiana* 'Crystal Bowl Purple'-CBP and 'Majestic Giant Red'-MGR. Asterix indicates significant difference at $P<0.05$. Arrows indicate the LT_{50} values for each cultivar. r^2 for MGR= 0.78, r^2 for CBP= 0.81.

Western Blotting

Western blotting to identify sHSP from leaf samples resulted in an increment in accumulation of HSP27 as the temperature increased in comparison to the control (25 °C) for both CBP and MGR (Fig 7.6 A&B). For both CBP and MGR, protein bands were observed starting at 30 °C but distinct bands were observed in CBP samples at 30 °C and at 35 °C for MGR. At 45 °C both the cultivars synthesized sHSP27 similarly. Above 30 °C protein bands were observed in both the cultivars, however at 40 and 45 °C bands were more distinct for CBP.

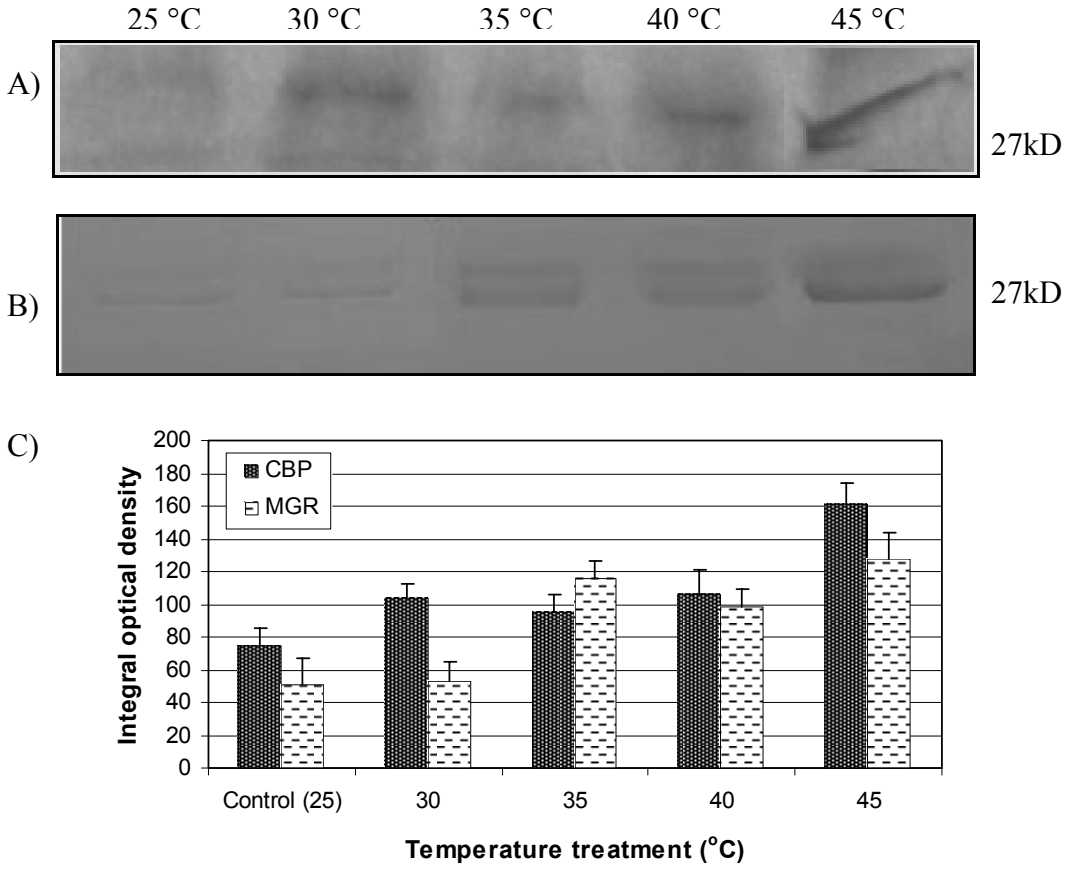

Figure 7.6.Effect of temperature treatments applied for 3h on accumulation of small heat shock protein 27kD in *Viola x wittrockiana* A) Crystal bowl purple and B)Majestic Giant Red- MGR C) Optical density values of bands measured with imajeJ software. Error bars represent mean of six measurements ±SE.

Marketable Quality

Overall marketable quality at the finish stage of plants declined as temperature treatment for both the pansy cultivars. CBP however maintained better marketable plants at minimum high temperature (30 °C) compared to MGR (Fig 7.7). A short duration exposure to 35 °C resulted in severe marginal burning of young plants (Fig 7.8A & B). At 35 and 40 °C neither of the cultivars was of marketable quality. A marketable quality score of seven and above was considered as accpetable.

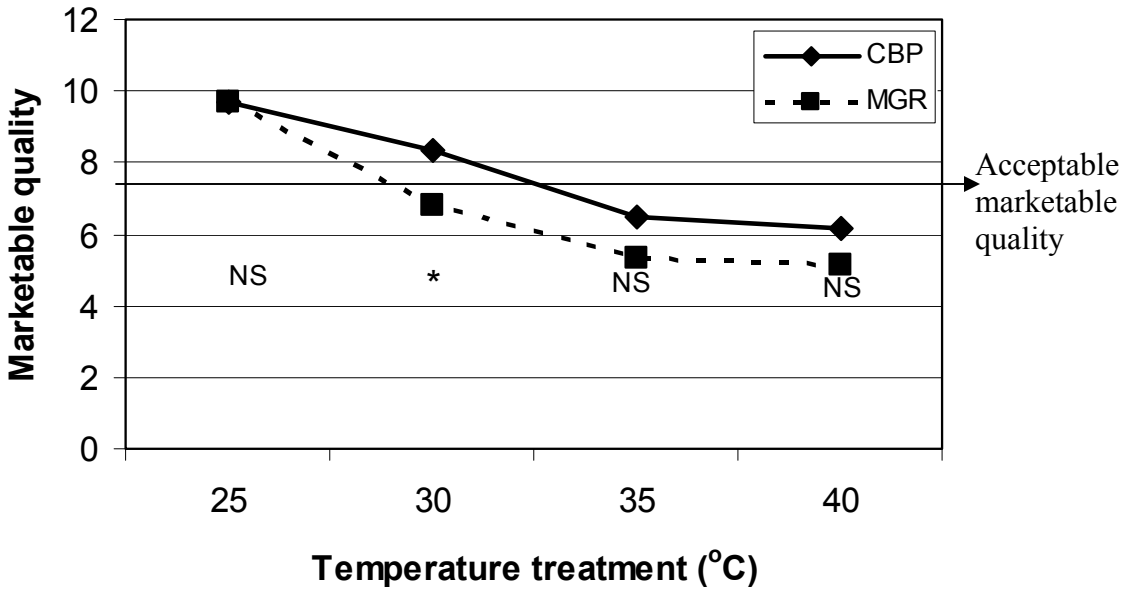

Figure 7.7. Effect of temperature treatments applied for 3h on marketable quality in *Viola x wittrockiana* cultivars Crystal Bowl Purple-CBP and Majestic Giant Red-MGR. Asterix indicates significant different at $P<0.05$ (Tukey's test). Quality score of 1=poor and 10= best.

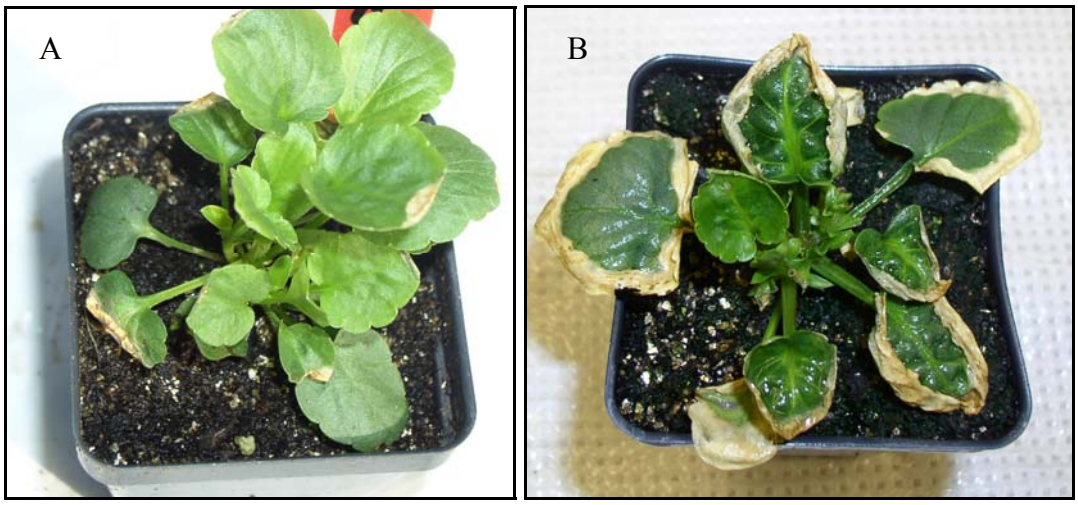

Figure 7.8: Marginal burning symptoms on *Viola x wittrockiana* leaves subjected to short duration high temperature stress of 35 °C for 3h every third day. A) Crystal Bowl Purple (CBP) B) Majestic Giant Red (MGR).

Experiment 2

Plant Growth

Heat preconditioning increased performance of both cultivars of pansy CBP and MGR at 30/23 °C as reflected by plant height (Fig 7.9A). Non preconditioned plants and subsequent growth in 30/23 °C, and both preconditioned and non preconditioned plants grown in 35/28 °C decreased in plant height for both CBP and MGR.

Preconditioned plants grown in 30/23 °C resulted in a maximum number of branches for CBP compared to the control and rest of the treatments (Fig. 7.9B). The number of branches per plant was significantly less for MGR compared to CBP with and without heat preconditioning and a subsequent high temperature treatment. As temperature treatment increased the number of branches decreased for both the cultivars above 30/23 °C.

Root dry weights increased for preconditioned CBP at both the challenging temperature compared to non preconditioned plants (Fig 7.10A). The maximum amount of root dry matter accumulation was noticed for preconditioned CBP grown at 30/23 °C treatment in comparison to all others. Both cultivars were severely affected when exposed to 35/28 °C without preconditioning treatment. Preconditioned MGR grown at 30/23 °C had equal root dry weight as that of control.

Shoot dry weight recorded in both the cultivars with preconditioning and subsequent growth at 30/23 °C was significantly greater when compared to plants at 35/28 °C with and without pre conditioning (Fig. 7.10B).

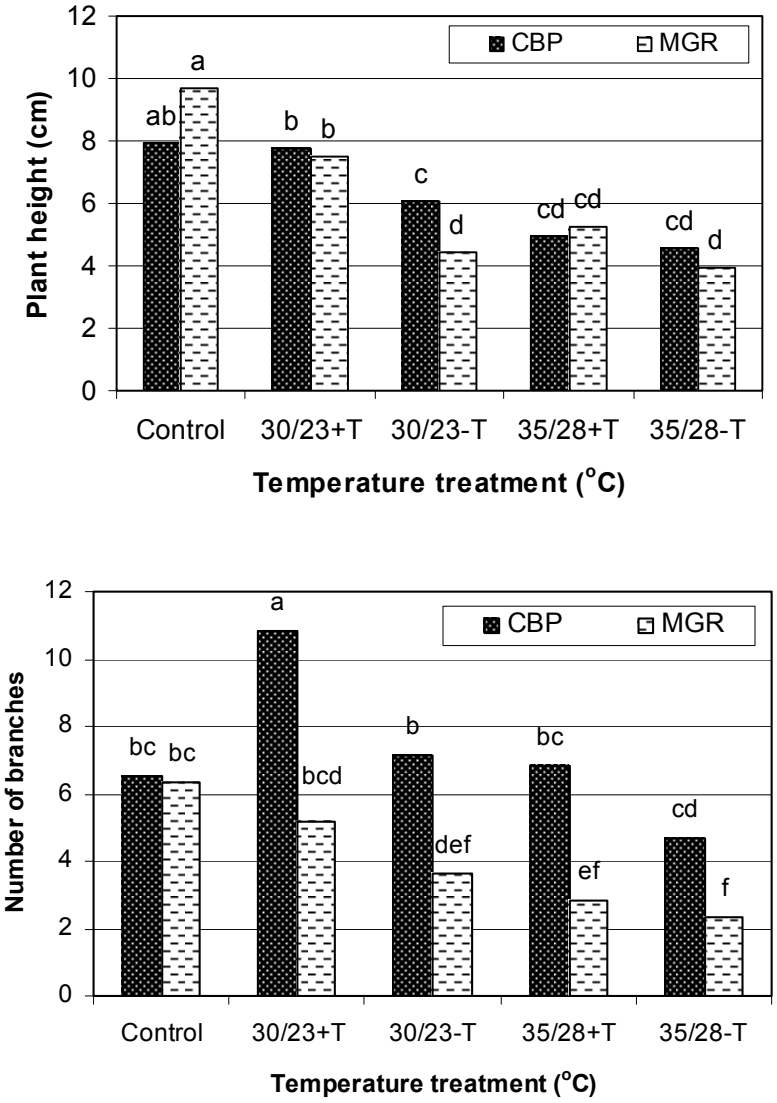

Figure 7.9. Effect of heat preconditioning (30 °C for 3h every third day for 4weeks) on A) Plant height B) Number of branches, in *Viola x witrrockiana* Crystal Bowl Purple - CBP (heat tolerant) and Majestic Giant Red-MGR (heat sensitive) when exposed to two challenging temperatures 30/23 °C and 35/28 °C. (+T heat precondition; -T no precondition. HT heat tolerant; HS heat sensitive). Means with different letters are significantly different at $P<0.05$ (Tukey's test).

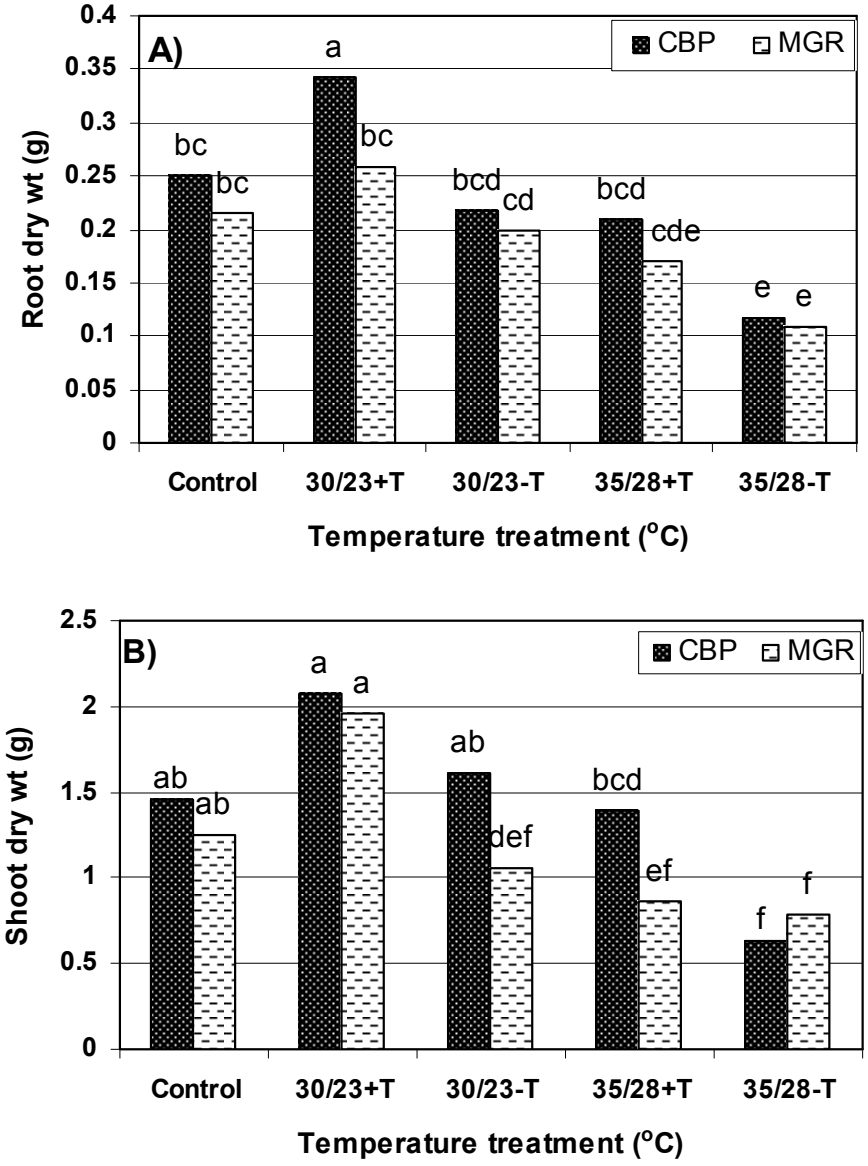

Figure 7.10. Effect of heat preconditioning (30 °C for 3h every third day for 4weeks) on A) Root dry weight B)Shoot dry weight, in *Viola x witrrockiana* Crystal Bowl Purple - CBP (heat tolerant) and Majestic Giant Red-MGR (heat sensitive) when exposed to two challenging temperatures 30/23 °C and 35/28 °C. (+T heat precondition; -T no precondition. HT heat tolerant; HS heat sensitive). Means with different letters are significantly different at $P<0.05$ (Tukey's test).

Transpiration (T), Stomatal Conductance (Gs) and Net Photosynthesis (Pn)

Transpiration rate was not much affected by high temperature treatments with and without heat preconditioning in both the cultivars (Fig 7.11A). Stomatal conductance improved to the level of control plants with heat preconditioning and subsequent growth at 30/23 °C for both CBP and MGR (Fig 7.11B). Plants grown at the higher temperature (35/28 °C) without preconditioning reduced the stomatal conductance for MGR when compared to control plants. Among the two cultivars no significant difference in stomatal conductance was obsereved at all the treatments. Net photosynthesis was greater at 30/23 °C with heat preconditioning for both CBP and MGR compared to plants grown at 35/28 °C with and without heat preconditioning (Fig 7.11C). For CBP heat preconditioning improved net photosynthesis at 35/28 C compared to non preconditioned plants at this high temperature. Non preconditioned plants grown at 30/23 °C and both preconditioned and non preconditioned plants at 35/28 °C for MGR showed no significant difference and Pn declined in comparison to the control and preconditioned plants grown at 30/23 °C.

Western Blotting

Small HSP of approximately 27kD were identified at all the temperature treatments irrespective of preconditioning treatment in leaf samples of pansy cultivars (Fig 7.12 A, B & C). For both CBP and MGR no distinct protein bands were identified in control samples. As the temperature treatment increased the intensity of bands increased but they are not significantly different, except for MGR with preconditioning and the 30/23 C challenging temperature.

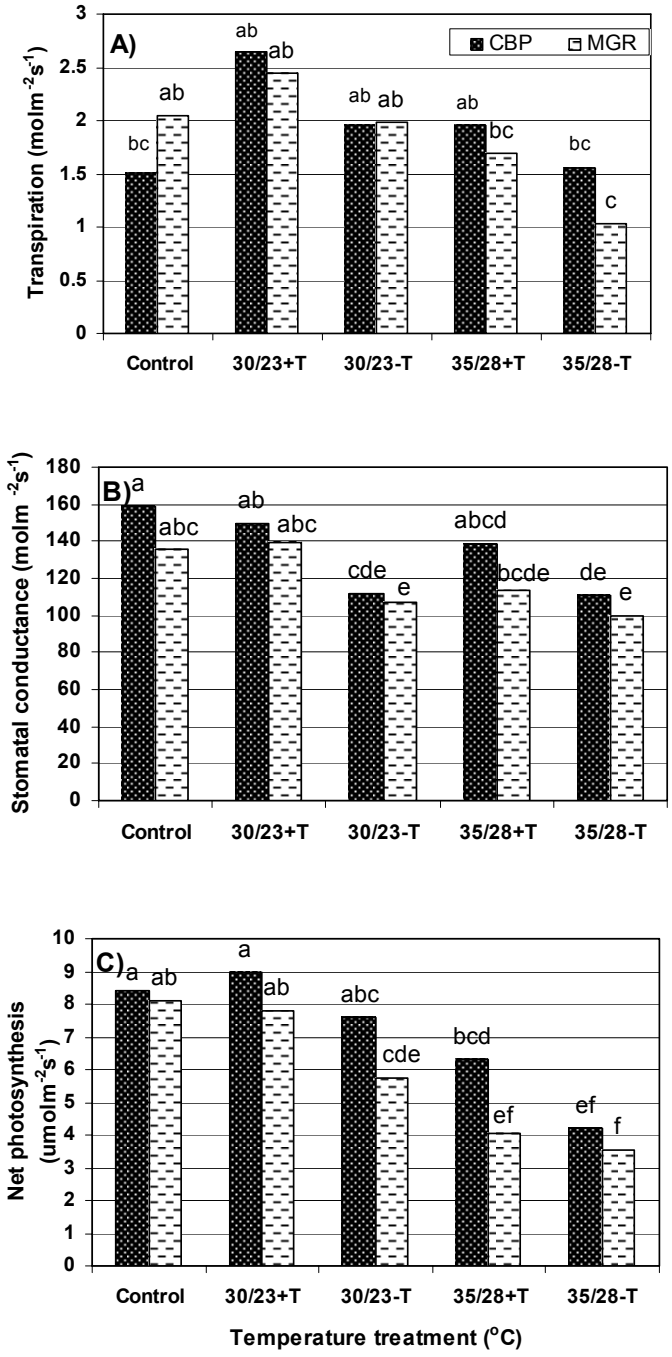

Figure 7.11. Effect of heat preconditioning (30 °C for 3h every third day for 4weeks) on A) transpiration rate B) stomatal conductance and C) net photosynthesis, in *Viola x witrrockiana* Crystal Bowl Purple -CBP (heat tolerant) and Majestic Giant Red-MGR (heat sensitive) when exposed to two challenging temperatures 30/23 °C and 35/28 °C. (+T heat precondition; -T no precondition. HT heat tolerant; HS heat sensitive). Columns with different letters are significantly different at P= 0.05. Means with different letters are significantly different at P<0.05 (Tukey's test).

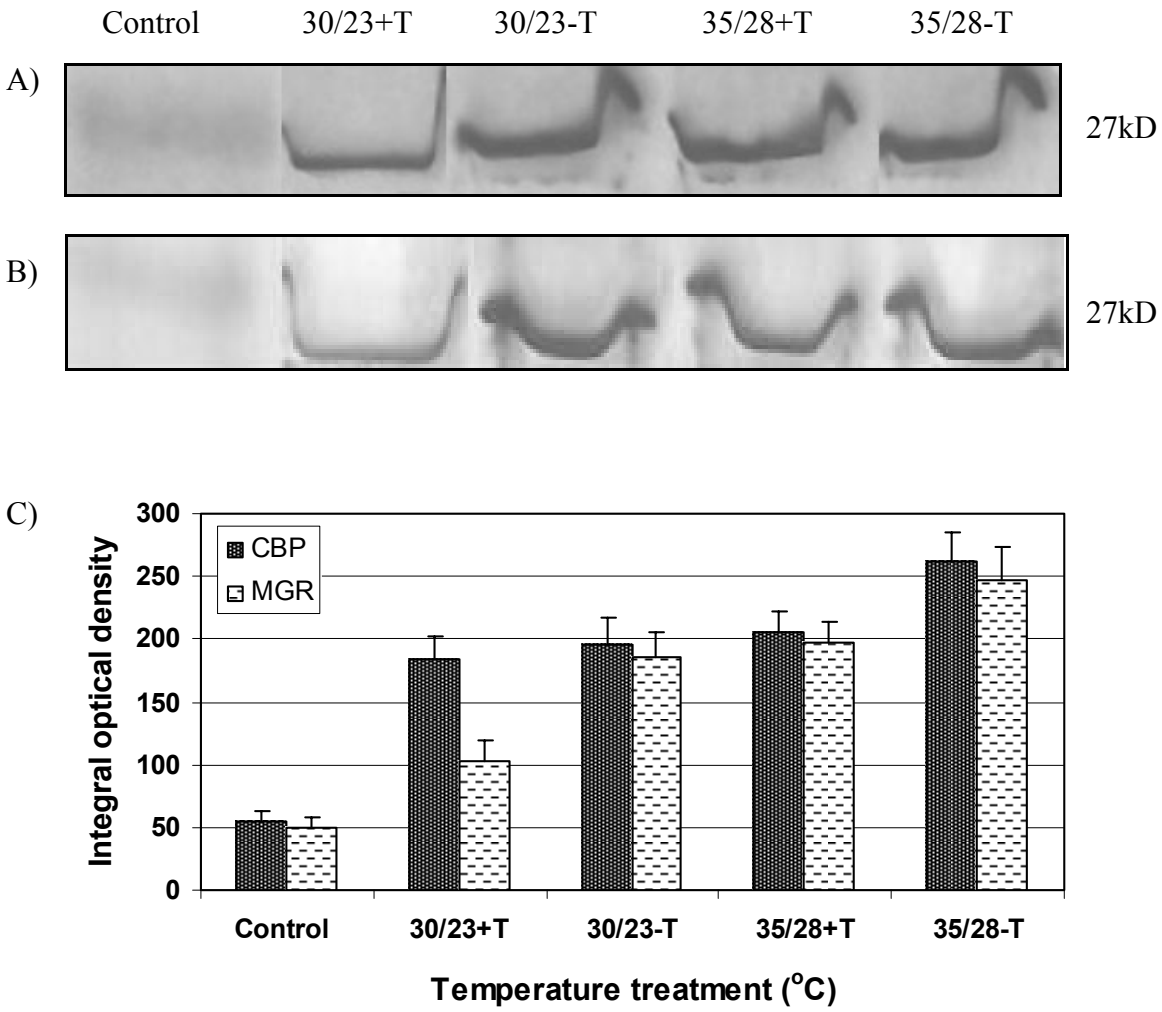

Figure 7.12.Effect of heat preconditioning (30°C for 3h every third day for 4weeks) and exposure to two challenging temperatures 30/23 °C and 35/28 °C. (+T heat precondition; -T no precondition. HT heat tolerant; HS heat sensitive) on synthesis of sHSP27, in *Viola x witrrockiana* A) Crystal Bowl Purple -CBP (heat tolerant). B)Majestic Giant Red-MGR (heat sensitive) C) integrated optical density values measured using ImajeJ software.Error bars indicates means of six observations ±SE

Days to Flower and Marketable Quality

Under control condition MGR flowered earlier compared to CBP (Fig. 7.13A). At the challenging temperature of 30/23 °C treatment DTF decreased for CBP with and without heat preconditioning. Preconditioning at both the challenging temperature, increased DTF in MGR. Without preconditioning MGR flowered earlier when compared preconditioned plants. Comparing the two preconditioned cultivars, CBP flowered a week earlier at 30/23 °C in comparison to MGR. Flowering delayed in CBP at 35/28 °C with and without the preconditioning treatment. As temperature treatment increased marketable quality of plants declined for both the cultivars (Fig. 7.13B). Heat preconditioning did improve marketable quality for MGR cultivars grown at the 30/23 °C treatment compared to non preconditioned plants. CBP with and without a heat precondition treatment and grown at 30/23 °C had better acceptable quality than MGR. Heat preconditioning showed no improvement in marketable quality of plants grown at 35/28 °C for both cultivars. Simple correlation of marketable quality with morphological traits showed that a positive correlation exists between plant morphological traits such as leaf area per plant, shoot and root dry weight. A decline in marketable quality of pansy at high temperatures are mainly due to leaf damage and poor root and shoot growth (Fig. 7.8 and 7.14).

Table 7.2. Correlation coefficients for association of marketable quality of pansy (*Viola x wittrockiana*) with morphological traits such as plant height, number of side shoots, leaf area per plant, shoot dry weights (SDW), and root dry weights (RDW).

	Plant height	No. of side shoots	Leaf area per plant	SDW	RDW
Marketable quality	38.5	37.5	53.6	75.37	67.72
P values	0.0069	0.0085	< 0.0001	< 0.0001	< 0.0001

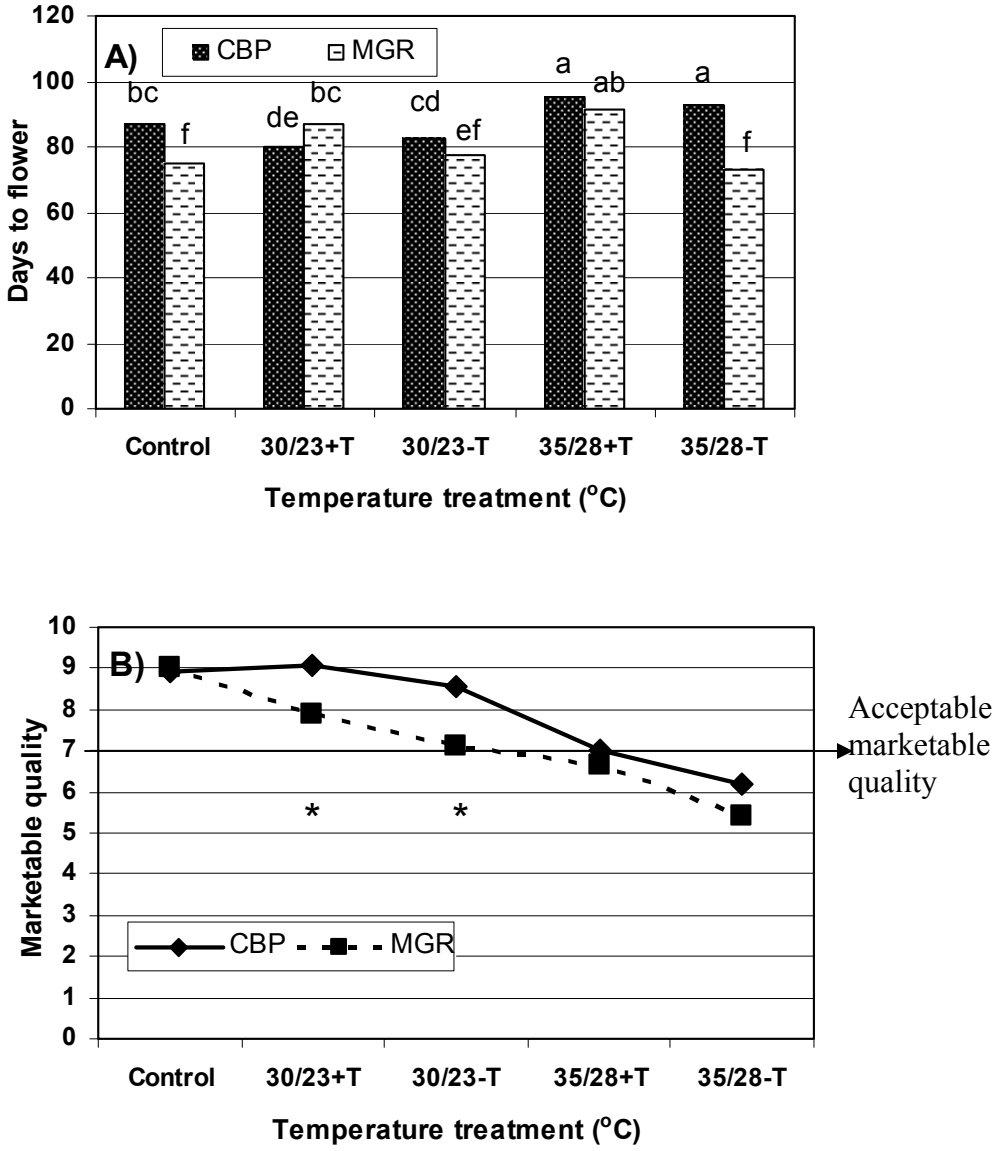

Figure 7.13. Effect of heat preconditioning (30 °C for 3h every third day for 4weeks) on A) days to flower and B)marketable quality, in *Viola x witrrockiana* 'Crystal Bowl Purple' -CBP (heat tolerant) and 'Majestic Giant Red'-MGR (heat sensitive) when exposed to two challenging temperatures 30/23 °C and 35/28 °C. (+T heat precondition; -T no precondition. HT heat tolerant; HS heat sensitive). Columns with different letters are significantly different at $P<0.05$. Means with different letters are significantly different at $P=0.05$ (Tukey's test). Asterix indicates significant difference between CBP and MGR. Quality score of 1=poor and 10= best.

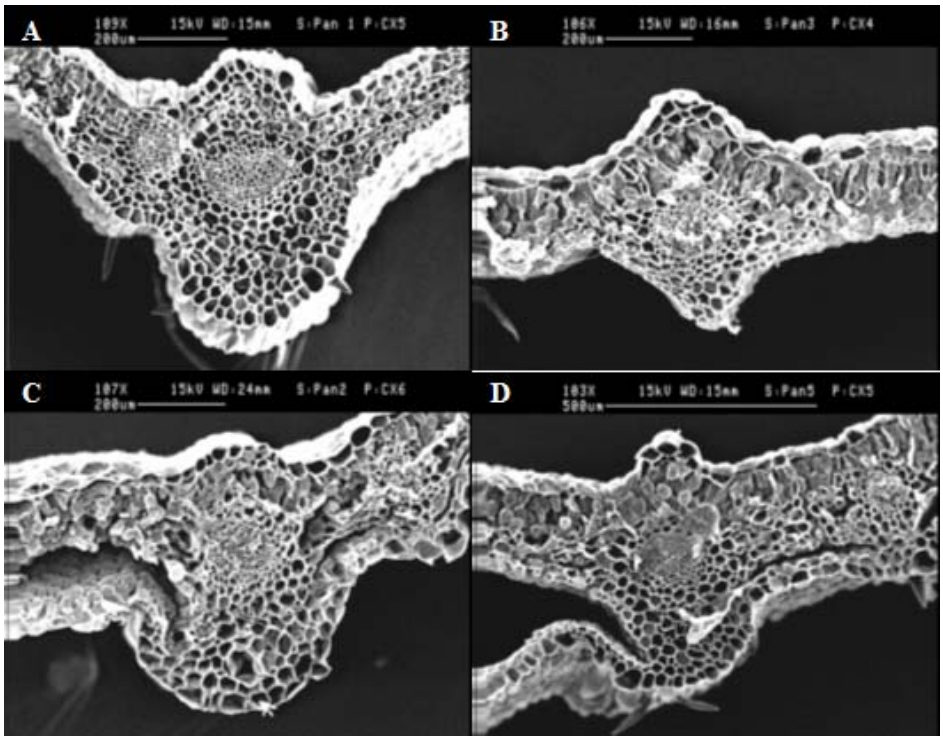

Figure 7.14. Leaf cross section SEM images (200X magnification) of *Viola x wittrockiana* cultivars A) 'Crystal Bowl purple'-CBP control B) 'Majestic Giant Red'-MGR control C) 'Crystal Bowl purple'-CBP (35/28 °C +T) and D) 'Majestic Giant Red'-MGR (35/28 °C +T) (+T Heat preconditioning at 35 °C for 3h every third day for 5 weeks).

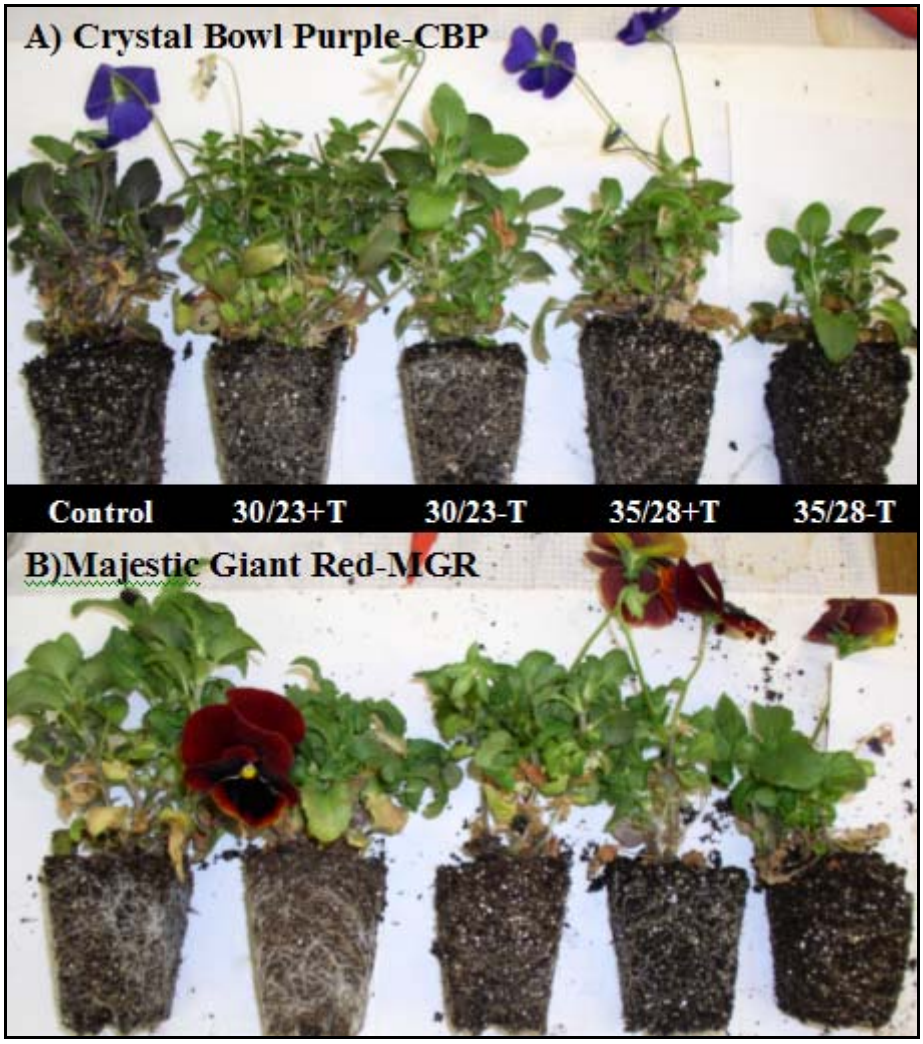

Figure 7.15. Effect of heat preconditioning (30 °C for 3h every third day for 4weeks) on overall growth and development, in *Viola x witrrockiana* 'Crystal Bowl Purple' -CBP (heat tolerant) and 'Majestic Giant Red'-MGR (heat sensitive) when exposed to two challenging temperatures 30/23 °C and 35/28 °C. (+T heat precondition; -T no precondition).

DISCUSSION

Data presented in this study clearly shows that plant growth and dry matter accumulation in pansy is largely affected by high temperature treatments. Similar conclusions were obtained for pansy (*Viola x witrrockiana*) 'Universal Violet', where plant growth in terms of relative growth rate is a function of temperature and light intensity (Adams et al., 1997). Previous studies with other herbaceous annuals such as groundnut (*Arachis hypogea*) (Ong, 1984) have shown that a mean diurnal air temperature regime of 36/25 °C imposed from sowing to maturity reduced the crop yield by 50% relative to those produced by plants grown at 27/17 °C.

Adams et al. (1997) reported that an increase in the number of leaves in pansy (*V. wittrockiana*) was linearly related to growing temperature up to a maximum of 28.8 °C. This increase in leaf number with mean daily temperature was reported in other species also: *Hibiscus rosa- chinensis* (Moe and Heins, 1990) and chrysanthemum (Karlsson, 1989). Similar results were obtained in the present study, where the increased leaf number might be related to an increase in branch number from 25 to 30 °C (Fig 7.1B) and a decrease in size of individual leaves for CBP at high temperatures (personal observation Fig 7.15).

Decline in shoot dry weights of pansy cultivar CBP (63 %) and MGR (42 %) were attributed to a decrease in root growth under heat stress. Previous studies have indicated that leaf injury under high temperature can be due to direct inhibition of root growth which affects water and nutrient uptake (Graves et al., 1991; Huang et al., 1991). Physiological effects associated with poor root growth are reduced supply of water which

is required to compensate for transpirational water loss. This lack of water affected shoot growth due to oxidative stress in creeping bent grass (*Agrostis palustris*) (Kramer, 1983; Huang and Xu, 2000; Huang et al., 2001).

Greater transpiration rates at 30 and 35 °C, greater stomatal conductance at 35 °C, and greater net photosynthesis at 30 °C of CBP was attributed to several interlinked morphological and physiological traits of this relatively heat tolerant cultivar when compared MGR. Heat tolerance of plants in large part could be a result of an increased heat stability of the photosynthetic apparatus and thermotolerance of PSII. Both of these photosynthetic apparatus are dependent on thylakoid membrane stability that varies widely among species and also varies according to acclimation of PSII to heat stress (Berry and Bjorkman, 1980; Weis and Berry, 1988). In the present study sensitivity of both cultivars to high temperature stress are evident from the shift in the CMT response curve (Fig. 7.5). There was a sigmoidal relationship between percent electrolyte leakage and increasing incubation temperatures. Similar results were reported in soybean (*Glycine max*) (Martineau et al., 1979), melons (*Cucumus melo* L) (Lester, 1985), citrus (*Citrus* species) (Ahrens and Ingram, 1988) and turfgrass species (Marcum 1998). Positive associations between membrane stability and CO_2 assimilation and in increase in grain yield under heat stress have been reported for two spring wheat (*Triticum aestivum* L) populations (Blum et al., 2001).

Decline in concentrations of soluble sugars was apparent from the reduced rates of net photosynthesis at 30 °C for pansy in this study. In tobacco (*Solanum tobaccum*) and cotton (*Gossypium* species) photosynthesis was limited at 35 °C because of reduced activity of Rubisco (Crafts-Brandner and Salvucci 2000). Heat tolerant CBP showed a

greater concentrations of sucrose and glucose at 30 °C compared to MGR. Jiao and Grodzinski (1996) indicated an increase in mobilization of sucrose and other soluble sugars at 40 °C in heat tolerant cultivars of salvia associated with osmolyte accumulation which increased heat tolerance.

At the molecular level plant heat tolerance was associated with an accelerated increase in sHSP. A significant differential expression pattern of chloroplast sHSP was reported in genotypes of *Agrostis stolonifer* differing in heat tolerance (Heckathorn et al., 2002). The possible role of HSPs in heat tolerance under heat stress condition of arabidopsis, several agronomic crops and turf grass species were presented in previous research (DiMascio et al.1994, Queitsch et al., 2000, Luo Jinn, 2004). For example a mutant arabidopsis for HSP failed to survive under 30/24 °C heat stress compared to wild type. In this study the sHSP of approximately 27kD was identified using antibodies and western blot technique. The integral optical density values measured showed a differential expression of 27 kD HSP in two cultivars of pansy.

Heat stress beyond a certain specific temperature of 37 °C resulted in perturbed physiological functions reducing plant growth and yield in two ecotypes of Red bud (*Cercis canadensis*) (Griffin et al., 2004). This critical temperature differs from species to species of plants (Law and Crafts-Brandner, 1999). Fom this study and previous studies in pansy cultivars the critical temperature was found to be between 25 and 30 °C day temperature.

Based on the results from experiment 1, the 30 °C temperature, identified critical day temperature (challenging temperature) for pansy and was used to study heat preconditioning to induce heat tolerance in the second experiment using the same

cultivars. All the plant growth parameters and gas exchange measurements were recorded to assess the inherent and induced heat tolerance in CBP and MGR.

Results show that prior exposure of plants to heat preconditioning before exposure to continued heat stress at two challenging temperatures improved plant growth and physiological parameters only at the lower challenging temperature 30/23 °C for both cultivars. Similar conclusions were obtained in Kentucky blue grass (*Poa pratensis*) where, preconditioning improved root growth, canopy photosynthesis and turf quality (Jiang and Huang, 2001). Senthil et al. (2003) reported the effects of preconditioning and subsequent challenging temperature on growth and survival of sunflower seedlings. Under optimal growing conditions CBP and MGR differ in terms of growth pattern (personal correspondence). While heat preconditioning improved plant growth to some extent in both cultivars grown at 30/23 °C challenging temperature, both preconditioned and non preconditioned MGR plants at 35/28 °C resulted in earlier leaf senescence, burning symptoms, less number of side branches (Fig.7.8&7.9B).

Damage to the leaf tissue at the high challenging temperature may be due to epidermal tissue damage (Fig.7.14) that resulted in severe necrosis and burning symptoms on leaves (Fig. 7.8B). CBP plants however, showed no severe symptoms of leaf damage even at high challenging temperature, but did have decreased number of flower buds. Dry matter accumulation in terms of root dry weight improved after preconditioning and subsequent growth at 30/23 °C for CBP, whereas MGR maintained equal root dry weight as that of control. Effect of challenging temperatures on shoot growth was evident from the root dry matter accumulation, where extensive root growth improved shoot growth after preconditioning and 30/23 °C heat stress. Similar

conclusions were obtained in earlier studies. Preconditioning of Kentucky blue grass (*Poa pratensis*) and subsequent growth at 35/30 °C improved the root dry weight and soluble carbohydrate accumulation compared to non preconditioned plants under heat stress (Jiang and Huang, 2001). Authors concluded that the enhanced heat tolerance of preconditioning was due to extensive root development, and osmotic adjustments due to accumulation of ionic solutes through roots and water soluble sugars.

Ahmad et al. (1989) suggested that heat injury is associated with water deficit and cell turgor. A heat tolerant cultivar of cotton was able to survive heat stress due to maintenance of turgor by accumulation of solutes and continuous water movement (Ashraf et al., 1994). A deep and extensive root system contributes tremendously to the plant water uptake (Huang and Fry, 1998; Bonos and Murphy, 1999). Physiological effects of preconditioning and subsequent heat stress were studied in cool season turfgrass cultivars differing in heat tolerance. Preconditioning by frequent soil drying improved heat tolerance in Kentucky blue grass (*Poa pratensis*) under 35/30 °C heat stress. They demonstrated that enhanced stomatal conductance and transpiration rate and significantly higher rates of net photosynthesis in preconditioned plants than non preconditioned plants (Jiang and Huang, 2000). Heat preconditioned CBP and MGR maintained greater stomatal conductance only at the 30/23 °C challenging temperature compared to non preconditioned plants, no significant difference was observed at 35/28 °C. Net photosynthesis however, improved only in preconditioned CBP. This could have been due to improved root growth and water relations as discussed ealier.

Another unique physiological response of plant in high temperature stress is synthesis of sHSP. Both cultivars with and without heat preconditioning produced small

HSP of approximately 27kD. Intensity of bands showed no significant difference in both cultivars in all high temperature treatments. At high challenging temperature of 35/28 °C sHSP synthesis was not found to be directly related to heat tolerance in pansy. Earlier studies reported that sHSP syntheses after preconditioning and heat tolerance were related. Precondtioned plants resulted in greater accumulation of sHSP18 under subsequent lethal high temperatures of 35/27 °C in wheat (*Triticum aestivum*) cultivars (Ozkan, 1995). They also reported that, preconditioning at 37 °C for 20h improved survival capacity of cultivars 'Tosun' and 'Karachia'. Study conducted in transgenic tomato (*Lycopersicum esculentum*) reported that suppression HSP synthesis expression resulted in poor survival capacity of plants even after heat preconditioning treatment. Whereas wild type plants and plants that overexpresed HSP synthesis survived under high temperature after preconditioning at 45 °C for 1h (Mishra et al., 2002). One possible reason for the absence of this relation in pansy could be growth habit of pansy. Pansy being a cool season bedding plants cannot withstand, a continuous heat stress of 35/28 °C which severely affected the growth of these plants. A study conducted by Park et al., (1997) in creeping bentgrass however, reported that the presence of a mere one to three additional HSPs from a multigene family of HSPs with diverse molecular weight classes would not likely result in increased heat tolerance.

Marketable quality of pansy declined with increasing temperature for both CBP and MGR. Preconditioning and subsequent growth at 30/23 °C decreased the number of days to flower with and without preconditioning for CBP and plants produced a maximum number of shoots before flowering. MGR flowered earlier at high temperature treatment and marketable quality deteriorated due to poor plant growth and decreased the

number of flowers. Adams et al. (1997) reported that the rate of progress in flowering for pansy (*Viola x wittrockiana*) cultivar 'Universal Violet' increased linearly with temperatures up to 21.7 °C. Beyond this temperature flower production in terms of size and number decreased. A High temperature treatment of 30 °C resulted in 80 % bud break in 'Hakuho' peach within 3 days when compared to 18 days for control plants (Kozai et al., 2004).

In conclusion high temperature treatment affected pansy growth and development under heat stress condition. Although pansy is one of the largest selling bedding plants, little was known about their physiological and morphological response to high temperatures. Also lack of laboratory techniques for screening pansy and other bedding plants rapidly and more efficiently is an another drawback for selection of heat tolerant cultivars. The present study has investigated all the morphological and physiological responses of pansy under high temperature. CMT response curves were shown to be an effective method for initial screening of pansy and other bedding plants with minimal amount of space and time. Cultivars with greater shoot growth parameters, such as greater number of side shoots and greater total leaf area per plant were correlated with marketable quality under high temperature. Gas exchange measurements such as Gs and Pn recorded at high temperature conditions clearly showed the physiological capability of pansy to survive under heat stress condition and this varies by cultivar. All of these traits hence could be used as "screening tool" for selecting heat tolerant plants. The ability of pansy to withstand high temperature seems to be limited and the critical or challenging temperature was determined to be 30 °C.

Heat preconditioning induced heat tolerance in relatively sensitive cultivars and enhanced heat tolerance in tolerant plants to a greater extent. The extent of heat tolerance induction was however, limited to moderate high temperatures (30/23 °C) for both the cultivars of pansy because of its cool season growth habit. Beyond this temperature growth and development deteriorated in both heat tolerant and heat sensitive cultivars. Hence this pretreatment before transplanting of bedding plants into landscapes could be used as preconditioning treatment to induce heat tolerance at production units by growers.

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CHAPTER 8. SUMMARY AND CONCLUSIONS

The effect of high temperature stress on popular bedding plants salvia (*Salvia splendens*) a warm season plant, and pansy/violas (*Viola x wittrockiana*) a cool season plant, was investigated to determine the various morphological and physiological responses associated with heat stress, heat tolerance and preconditioning. Heat tolerance is associated with the genotypic and phenotypic characteristics of plants. There is no information in regards to key morphological and leaf anatomical traits or physiological adjustment mechanisms of most series or cultivars of bedding plants to tolerate heat stress. Two different cultivars of salvia and pansy that were reported to differ in their heat tolerance were used in this study: salvia 'Vista Red' (heat tolerant) and 'Sizzler Red' (heat sensitive) pansy 'Crystal Bowl Purple' (heat tolerant) and 'Majestic Giant Red' (heat sensitive).

A short duration heat stress response was investigated starting at the seedling or plug stage and continued to finished or flowering plants for both the plant species. Morphological responses in terms of plant growth and dry matter accumulation significantly differed among the cultivars studied. Heat tolerant cultivars acclimated to short duration heat stress by acquiring and or enhancing certain already existing traits such as shortening of internodes, thickening of leaves, delay in flowering, increased stomata number per unit area, development of cuticular thickenings on leaf abaxial surface, increased thickness of mesophyll layer and well developed vascular bundle are observed in heat tolerant cultivars. Such traits are usually found in plants that are adapted to high temperature conditions. Heat sensitive cultivars attained these phenotypic traits, but only at moderate heat stress condition.

Heat tolerant cultivars maintained a greater transpiration rate and stomatal conductance. Transpiration is the only physiological mechanism in plants that enable them to adjust their tissue temperature significantly and thus results in lesser damage to the photosynthetic apparatus due to heat stress. Increased leaf thickness and stomatal conductance improved the CO_2 assimilation capacity of plants that might have compensated respiration losses of food reserves due to high temperature. Soluble sugars such as raffinose and sucrose concentrations increased significantly in heat tolerant cultivars of salvia at high temperatures. These sugars probably act as osmoprotectants under high temperature conditions and are believed to be involved in stabilization of membranes.

Cell membrane stability measurements using an electrolyte leakage technique facilitated the determination of heat tolerant cultivars at the cellular membrane level. Lesser membrane damage in heat tolerant cultivars compared to non heat tolerant cultivars could be attributed to greater stability of cellular and organelle membrane. Another possibility for the greater membrane stability might be due to lesser phase change from liquid crystalline to solid gel state of the lipid bilayer at high temperature.

A primary molecular response of plants to heat stress is accelerated increase in heat shock proteins synthesis. In the present study this response was investigated to identify if any HSPs are involved in heat tolerance of salvia and pansy. A small heat shock protein of approximately 27kD, one of the small heat shock proteins from a large class of HSP was identified. Synthesis of these proteins increased with increasing temperature treatment. Expression of these proteins was greater in heat tolerant plants compared to non heat tolerant plants. This suggested that heat tolerance could be due in

part to expression of these proteins which might be acting as molecular chaperones to protect certain heat sensitive biomolecules.

From these initial experiments of this research project it was concluded that certain morphological traits such as leaf characteristics and stem characteristics, or physiological traits such as stomatal conductance and photosynthesis measurements could be used effectively for selecting heat tolerance of salvia and pansy. CMT measurements could probably be effectively used as screening tool for rapid and inexpensive screening of a large number of cultivars under laboratory condition. Identification of sHSP could be used for screening plants at the molecular level but the primary disadvantage for its application is in depth of sample preparation and analysis.

Further studies to investigate the effects of heat preconditioning to induce heat tolerance in bedding plants were conducted. Two week old salvia seedlings were heat preconditioned at 35 °C every third day until plants were five weeks old. Preconditioned plants subsequently grown at two challenging temperatures 30/23 °C and 35/28 °C resulted in improved growth in terms of shoot and root dry matter accumulation, greater transpiration, stomatal conductance, and net photosynthesis. This response was most significant in the heat sensitive cultivar ‘Sizzler Red’. The marketable quality of plants that received the precondition treatment improved over non preconditioned plants under heat stress conditions. Similar results were obtained for pansy cultivars after heat preconditioning of four week old seedlings at 30 °C. Precondition was only effective at 30/23 °C challenging temperature and the 35/28 °C challenging temperature was found to be too extreme for this cool season crop. The results from these experiments confirm previous conclusions from various studies that a brief exposure of plants to sub lethal

heat stress (heat precondition) develops the ability of plants to withstand severe high temperatures. This phenomenon is commonly referred as acquired thermo tolerance.

Expression sHSP 27 was observed in heat preconditioned and non preconditioned plants at both challenging temperatures; however there was no consistent relationship between expression of these proteins and acquisition of thermotolerance in heat preconditioned plants of both salvia and pansy.

Finally a screening test using CMT measurement was refined by modifying the technique of obtaining CMT response curves based on previous research. Instead of recording electrical conductivity (EC) at increasing incubation temperatures requiring a large number of water baths, a single high temperature exposure with increase in exposure time was investigated. Salvia cultivars previously used in this study along with a relatively more heat tolerant salvia (*Salvia coccinea*) 'Lady in Red' to test CMT measurements. CMT response curves were compared with leaf relative water content (RWC), gas exchange measurements, and marketable quality of plants under a heat stress (35/28 °C) condition. The CMT response curve of 'Lady in Red' was equal to that of 'Vista Red'. When compared to 'Sizzler Red', both cultivars had less damage to cell membranes in terms of percent electrolyte leakage. Leaf RWC in 'Vista Red' and 'Lady in Red' under heat stress condition showed no significant difference compared to control. While 'Sizzler Red' had decreased RWC starting at the sixth week and decreased below the control level as the number of weeks of exposure increased. Net photosynthesis was also significantly greater in 'Vista Red' and 'Lady in Red'. Overall marketable quality of plants showed similar trends.

Future research should be conducted on a large number of series and cultivars of bedding plants using the single temperature CMT technique for determination of heat tolerance. Morphological, anatomical and physiological traits should be investigated on these plants found to be highly heat tolerant to determine the appropriate genotypic responses to heat tolerance for breeding purposes. Further research on heat preconditioning via temperature and/or plant growth regulator treatments should be investigated for acclimation of bedding plants to high temperatures at plug transplant and landscape transplant. Two critical points in bedding plant production.

VITA

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